CHEMICAL REVIEWS

REVIEW

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Enantioenriched Compounds via Enzyme-Catalyzed Redox Reactions

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CONTENTS

1. Introduction 4088
2. Techniques, Biocatalysts 4089
   2.1. Reduction 4089
      2.1.1. Stereoinversion 4089
      2.1.2. Dynamic Reductive Kinetic Resolution 4090
      2.1.3. Asymmetric Reduction of Prochiral Compounds 4091
         2.1.3.1. Alcohol Dehydrogenases 4091
      2.1.4. Outlook 4097
   2.2. Oxidation 4097
      2.2.1. Deracemization 4097
      2.2.2. Asymmetrization of Meso Diols 4097
      2.2.3. Desymmetrization 4098
         2.2.3.1. Monooxygenases 4098
      2.2.4. Outlook 4100
   2.3. Engineering Techniques 4100
3. Cofactor Regeneration 4101
   3.1. Alcohol Dehydrogenase 4101
   3.2. Formate Dehydrogenase 4101
   3.3. Phosphite Dehydrogenase 4102
   3.4. Hydrogenase 4102
   3.5. Pyridine Nucleotide Transhydrogenase 4102
   3.6. Glucose Dehydrogenase 4103
   3.7. Amino Acid Dehydrogenase and Amino Acid Oxidase 4103
   3.8. NADH Oxidase 4103
   3.9. Others 4104
4. Case Studies 4105
   4.1. Montelukast 4105
   4.2. Lipitor 4106
   4.3. Crixivan 4106
   4.4. (*R*)-2-Methylpentanol as Building Block 4106
Author Information 4106
Biographies 4106
References 4107

1. INTRODUCTION

Redox reactions are essential to any currently known form of life and are at the core of a majority of metabolic processes, such as cellular respiration or photosynthesis. As about 25% of known enzymes are oxidoreductases,[1] it is no wonder that chemists have taken advantage of redox reactions occurring in Nature to serve synthesis purposes.

While the pharma industry is driven by the need for enantiopure drugs (the majority of small molecule active pharmaceutical ingredients (APIs) having at least one chiral center are developed as single enantiomers[2]), there is still a lack for broad synthesis methods that would generate both enantiomeric forms of a target compound in high purity. Homogeneous catalysis and organocatalysis recently have shown great progress toward this goal,[3−5] but the past decade has seen even more advances from biocatalysis. Enzymes are biological catalysts and their use is less subjected to environmental regulations and societal scrutiny. Biocatalysts not only commonly work under ambient conditions of temperature, pH value, and pressure in aqueous solutions, a greener approach to chemistry but, most importantly, are highly chemo-, regio-, and stereoselective and therefore of great interest in fine chemical synthesis.

Oxidoreductases constitute EC class number 1 and are classified into 22 subclasses. They allow the creation of stereogenic centers or the enantioenrichment of existing ones. While abundant literature has been published on biocatalyzed redox reactions, it is essential to stress their applicability in asymmetric synthesis. Thus, this review highlights the tremendous progress achieved over the last 10 years in the application of biocatalysts in the synthesis of enantiomerically pure compounds (EPCs), whether in purified form or as whole-cell biocatalysts, while specifically emphasizing on applications for the pharma industry. Advances in protein engineering are the main cause of that progress. It is now common to encounter enzymes with a broad substrate spectrum on non-natural substrates while displaying high chemo-, regio-, and stereoselectivity.

This review summarizes major advances dealing with reductases and oxidases and is organized according to important concepts in asymmetric biosynthesis. Therefore, advances that do not employ oxidoreductases, such as the transaminase-catalyzed synthesis of sitagliptin, the active ingredient of Januvia and Janumet, are not covered by this review. The review highlights specific examples where the use of redox biocatalysts has allowed the development of synthetic routes to pharmaceutically relevant molecules by enhancing the optical purity of the product,

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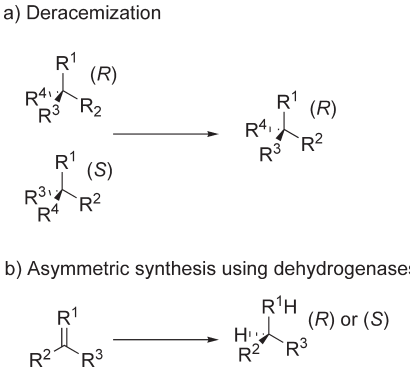

Figure 1. General concept in reductive processes for access to enantiopure compounds from (a) a racemic mixture and (b) a prochiral compound.

giving access to previously nonaccessible enantiomers, or increasing space-time yields and industrial applicability.

2. TECHNIQUES, BIOCATALYSTS

2.1. Reduction

The production of optically active compounds integrating redox biocatalysis has dramatically increased over the past decade. The synthesis of pure enantiomers or diastereomers using reduction reactions can proceed through the use of various chiral technologies (Figure 1), starting either from a racemic mixture of the desired product (stereoinversion or dynamic reductive kinetic resolution), or from a prochiral compound where chiral centers are introduced via selective reductive reactions (asymmetric synthesis using dehydrogenases). These technologies are presented here in their respective categories, while focusing on the most recent examples and their relevant applications in modern pharma industry.

2.1.1. Stereoinversion. Stereoinversion allows the optical enrichment of a racemate into a single enantiomer (deracemization) as well as the transformation of a pure enantiomer into the opposite enantiomer (Figure 2). Thus, strictly speaking, there is no net product formed. Stereoinversion traditionally combines concomitant enantioselective oxidation and asymmetric reduction, where the reduction step should not only be highly stereospecific but also irreversible to allow high enantiomeric excess. This technology has been widely applied to the production of optically pure secondary alcohols using alcohol dehydrogenases (ADHs), which work reversibly and can therefore catalyze both oxidation and reduction reactions.[6]

For long, this methodology was applied using whole-cells (fermenting or resting),[7−9] so that multiple parameters, such as enantiopurity, could not be controlled at the enzymatic level to adjust the outcome of the reaction. Despite this major drawback, this method has proved useful and allowed both stereoinversion and deracemization of alcohols. *Geotrichum candidum* for instance was employed with various arylethanols and both racemate and (*S*)-enantiomer could be converted to the (*R*)-enantiomer in high yield and high optical purity.[10,11]

The use of purified enzymes has dramatically improved the control of such processes, as various parameters in the whole reaction can now be tuned and adjusted to reach the desired outcome. Recent cases of stereoinversion using alcohol dehydrogenases have been developed by Kroutil et al. and have been reviewed.[12] In one example, lyophilized *Rhodococcus* spp. cells

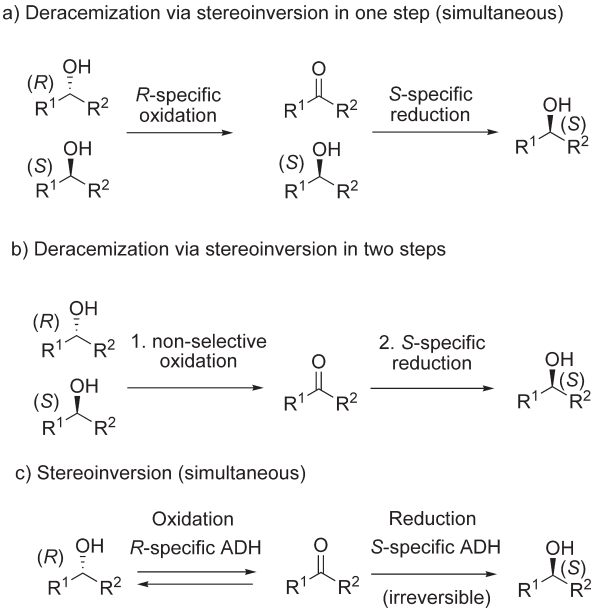

Figure 2. Stereoinversion/deracemization technologies using alcohol dehydrogenases (ADHs) to transform one enantiomer into the opposite optical form.

were employed, showing poor enantioselectivity in the oxidation reaction while they were highly stereospecific in the reduction reaction. Thus, the racemate (decan-2-ol) was oxidized to the corresponding ketone in the first step, and the second step was initiated by the addition of an excess of propan-2-ol to shift the reaction to the reductive mode. Overall, high yield and enantiomeric excess (*ee*) were obtained (82% and 92%, respectively).[13] It was not clear whether several ADHs were at play simultaneously.

In another example, stereoinversion of (*R*)-2-octanol was completed within 6 h by combining resting cells of *Alcaligenes faecalis* (catalyzing the enantioselective oxidation of the (*R*)-alcohol to the corresponding ketone) with an alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH, catalyzing the stereospecific reduction of the ketone to the (*S*)-alcohol) and a recycling system (glucose dehydrogenase and glucose). The same protocol (one-pot, one-step) could be applied to the efficient deracemization of *sec*-alcohols, and both enantiomers could be obtained in pure form by carefully choosing the microorganism and the ADH. Compartmentalization was necessary to avoid reversible reactions through consumption of both cofactor forms by the same enzyme and was provided by use of resting cells for one reaction, while the second simultaneous reaction took place with the isolated enzyme in the buffer.[14]

Lastly, a similar approach was applied using exclusively isolated enzymes. A four enzyme system consisting of an NADPH-dependent (*R*)-selective ADH, an NADH-dependent (*S*)-selective ADH, an NADPH oxidase (YcnD), and an NAD^+-specific formate dehydrogenase FDH (the latter two for cofactor recycling; see sections 3.2 and 3.8 for details on cofactor regeneration using FDH and NADH oxidase, and reviews on concepts for nicotinamide cofactor regeneration[15,16]) allowed the complete stereoinversion of (*R*)-octan-2-ol in three hours (Figure 3). Deracemization was also successful and *rac*-1-phenylethanol (50 mg) was converted to enantiopure (*S*)-1-phenylethanol in 90% isolated yield (45 mg, >99% *ee*) in a one pot

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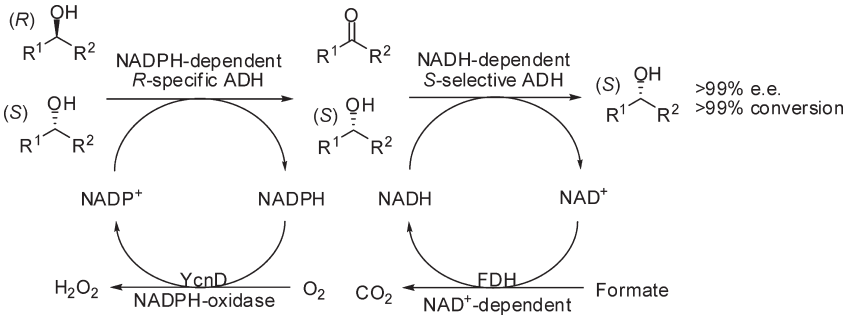


Figure 3. Deracemization via stereoinversion combining simultaneous concurrent tandem oxidation and reduction cycles with cofactor regeneration systems. Adapted with permission from ref 17. Copyright 2009 American Chemical Society.

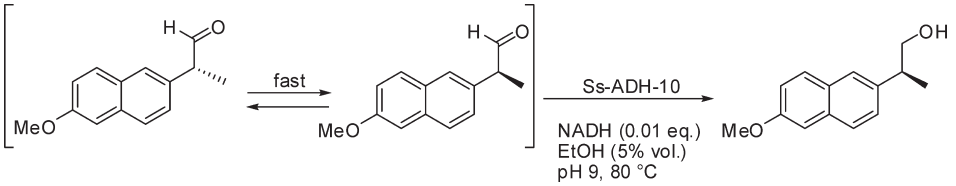


Figure 4. Entry to (*S*)-profens using dynamic kinetic reductive resolution DKRR. Adapted with permission from ref 21. Copyright 2010 American Chemical Society.

reaction (compartmentalization not necessary due to different cofactor specificities).[17]

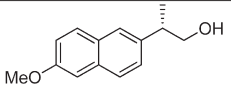
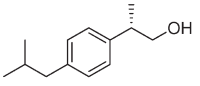
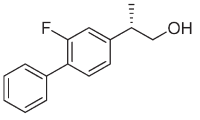
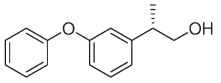
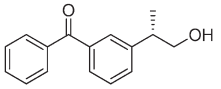
2.1.2. Dynamic Reductive Kinetic Resolution. The use of dynamic kinetic resolution (DKR) to purify racemic mixtures and thus provide pure enantiomeric products relies on in situ racemization (usually via transition metal-based catalysis or use of racemases) combined with selective transformation of one single enantiomer. While this method is powerful and easily allows one to overcome the major shortcoming in kinetic resolution (yields limited to 50%), it cannot traditionally be applied to bioreduction processes and the preparation of enantiopure alcohols for instance as the starting materials (the corresponding carbonyl compounds) would not have any racemization counterparts.

Examples exist however where this was successfully applied to α-alkyl-1,3-diketones and α-alkyl-β-keto esters, with advantage taken of the equilibrium between the two enantiomers of the starting material in aqueous buffer[18,19] (see recent review for additional examples[20]).

An elegant way to implement DKR with reductive biotransformation was recently shown with chiral aldehydes bearing an α-stereocenter and leading to entry to (*S*)-profens via (*S*)-profenols.[21] The enzyme employed was alcohol dehydrogenase SsADH-10, one of 13 annotated ADHs in the hyperthermophile *Sulfolobus solfataricus*. Racemic 2-arylpropionaldehydes were readily racemized under the experimental conditions used for the biotransformation (pH 9, 80 °C), which could evidently be sustained by the geothermal dehydrogenase. High yields (up to 99%) along with high enantiomeric excess (up to 99%) were obtained through the ADH-catalyzed reduction of the aldehyde moiety in the preparation of Profen drug precursors, that could be used for the synthesis of various nonsteroidal anti-inflammatory drugs (NSAIDs, Figure 4 and Table 1).

A similar process has been investigated in the past employing whole cells of Baker's yeast and 2-methyl-3-oxopropionate as substrate (Figure 5). Since the latter exists as a mixture of the aldehyde and the enol form, interconversion between the two enantiomers was fast and yielded a racemic mixture for the reduction reaction. *Ee* values depended on the ester alkyl chain

Table 1. Conversion of Profen Drug Precursors via DKRR[21]

(*S*)-profenols and corresponding derived NSAIDs	Time (h)	Conversion (%)	E.e. (%)
 Naproxen	18	96	98
 Ibuprofen	18	92	99
 Flurbiprofen	18	99	90
 Fenoprofen	12	85	95
 Ketoprofen	18	85	95

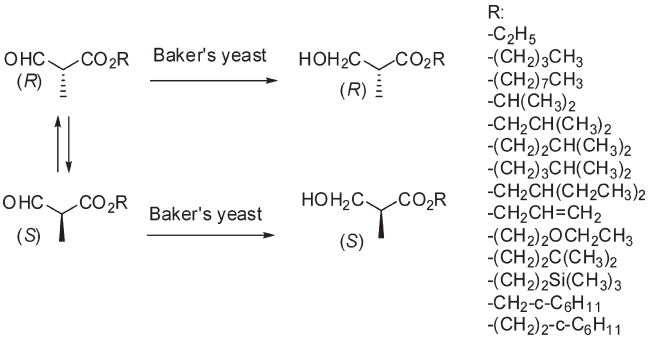


Figure 5. Reduction of 2-methyl-3-oxopropionate with baker's yeast. Adapted with permission from ref 22. Copyright 1988 CSJ.

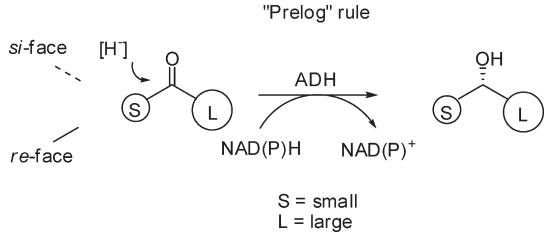


Figure 6. "Prelog" rule in the bioreduction of carbonyl compounds with ADH. In this example, the enzyme follows the Prelog rule with hydride attack from the *re*-face. Adapted from ref 28 with kind permission from Springer Science + Business Media: Biotransformations in Organic Chemistry, 2004, Faber, K., Scheme 2.118.

and did not exceed 90% at the best, which can be explained by the use of whole cells as catalysts and thus by the possible presence of several dehydrogenases.[22]

2.1.3. Asymmetric Reduction of Prochiral Compounds. Prochiral substrates can be reduced to optically pure compounds using dehydrogenases. Most common examples include the reduction of carbonyl groups of ketones (alcohol dehydrogenases) or the reduction of carbon−carbon double bonds (ene-reductases or enoate reductases). Other reductases have also proved efficient in stereoselective reactions (aldehyde reductases, diketoreductases, amino acid dehydrogenases, aldoketoreductases).

2.1.3.1. Alcohol Dehydrogenases. Alcohol dehydrogenases (ADHs, EC. 1.1.1.1) are among the most investigated redox enzymes and have been developed extensively to the point where they are now used in multiple industrial processes. They are employed in the enantioselective reduction of various nonnatural ketones to produce mainly enantiopure secondary alcohols.[23−25] These enzymes are essentially NAD(P)H-dependent enzymes and the need for (expensive) nicotinamide cofactor has led to extensive research in cofactor recycling (see section 3).[26,27] The enzymatic mechanism is well understood and the reduction of carbonyl compounds proceeds through hydride attack on either the *si*- or *re*- face of the substrate, leading to either (*S*)- or (*R*)-alcohol, while the enzyme transfers either the pro-(*R*)- or pro-(*S*)-hydride of the nicotinamide cofactor (total of four possible pathways). The "Prelog" rule allows prediction of the stereochemical outcome of the reaction based on the size of the substituents on the carbonyl group (Figure 6).[28] The enantiopurity of the product is usually higher when the two substituents are large and small respectively, allowing better stereorecognition by the enzyme, though advances in protein engineering will most likely render this requirement less important.

For instance, C295 of *sec*-ADH from *Thermoanaerobacter ethanolicus* (SADH) was subjected to mutation and was shown to be part of a small alkyl group binding pocket whose size determines the binding orientation of ketone substrates (i.e., also the stereochemical configuration of the alcohol product). C295A SADH was shown to have much higher activity toward *t*-butyl and some α-branched ketones than did the wild type and showed good to excellent stereoselectivity on ethynylketones, such as 4-methyl-1-heptyn-3-one. Even a switch of stereopreference on substrates of intermediate size could be obtained.[29]

In another example, one single-point mutation dramatically changed the substrate specificity of a *sec*-ADH from *Thermoanaerobacter ethanolicus* 39E (TeADH).[30] The W110A mutant accepts aryl derivatives of phenylacetone and benzylacetone and produced (*S*)-4-phenyl-2-butanol in >99% *ee*. Furthermore, this result is valuable for industrial application because of the high thermal and solvent stability of the enzyme.

Also, screening of microorganisms has revealed the existence of strains capable of reducing bulky−bulky aryl alkyl substrates in a highly stereospecific manner.[31,32] Recently, the strain *Nocardia globerula* DSM 46019 was shown capable of reducing bulky prochiral ketoesters to their corresponding hydroxyesters with high enantioselectivity.[33] The enzyme responsible for the catalysis (NGADH) was identified as a short chain alcohol dehydrogenase/reductase (SDR) related to the family of 3-hydroxyacyl-CoA dehydrogenases. Various bulky−bulky ketones such as 3-, 4-, or 5-decanone and their homologues, and ketoesters were converted and the enzyme displayed high stereospecificity against substrates such as ethyl 2-oxo-4-phenylbutanoate and ethyl 4-chloro-3-oxo butanoate (corresponding alcohols produced in >96% and >99% *ee*, respectively), while selectivity with 2-octanone was poor ((*R*)-octanol produced in 40% *ee*). Ten millimolar ethyl 2-oxo-4-phenylbutanoate was almost fully converted (95%) in 20 min using a cofactor regenerating system.

Additionally, new enzymes are being identified, isolated and characterized,[34−36] which contributes to further broaden the toolbox available to chemists to produce optically active alcohols.

2.1.3.2. Ene-reductases. The reduction of carbon−carbon double bonds using enzymes has long been attracting the interest of chemists for its high potential in creating two stereogenic centers in one reaction. Homogeneous catalysis was the method of choice until recently (classic examples are the Monsanto process using Rh-catalyzed hydrogenation with chiral diphosphines and the Noyori asymmetric hydrogenation with Ru complexes bearing chiral phosphines),[3,4,37] while organocatalysis is now showing some remarkable chemistry.[38−40]

For long, one of the only examples of biocatalyst capable of performing such reaction was the Old Yellow Enzyme OYE (EC 1.6.99.1) from yeast, discovered almost 80 years ago. In the early 1980s, while biochemists were further investigating OYE and its homologues, Simon et al. published on "true" enoate reductases (EC 1.3.1.31) from anaerobic *Clostridium* species (*C. kluyveri*, *C. tyrobutyricum*, and *C. sporogenes*) catalyzing the reduction of carbon−carbon double bond of α,β-unsaturated carboxylate anions.[41−43] Though belonging to different subclasses of oxidoreductases, OYE-like enzymes and enoate reductases provide the same appeal to modern chemists, as up to two stereogenic centers are created through the reaction. Enoate reductases, however, are mostly found in anaerobic organisms and thus present some drawbacks from an engineering point of view. They

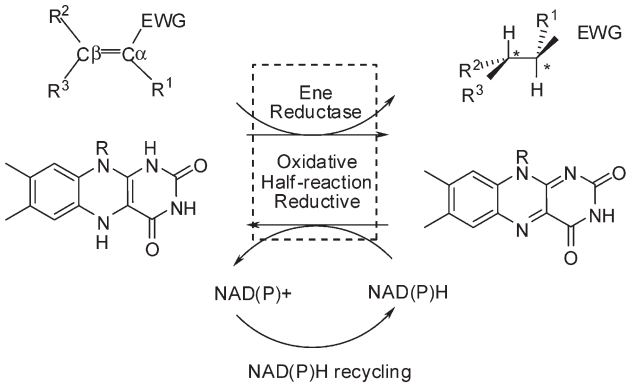


Figure 7. Asymmetric reduction of α,β-unsaturated compounds by ene-reductases from the OYE family. Adapted from ref 44 with permission. Copyright 2007 Elsevier.

represent fewer examples than OYEs and these are presented at the end of the section.

Old Yellow Enzyme Flavoproteins. While the OYE family of flavoproteins catalyzes the reduction of various compounds such as nitro esters, nitroaromatics or α,β-unsaturated compounds, the latter example is of greater interest for synthetic purpose. The reduction of activated carbon−carbon double bonds with OYEs at the expense of NAD(P)H proceeds indeed in a highly stereoselective fashion. Though OYE from yeast has been around for almost 80 years, it is only recently that the use of OYE homologues has shown their full potential for the asymmetric synthesis of various chiral intermediates.

The reaction proceeds through a Michael-type addition of $[H_2]$ onto a C=C bond, following a ping-pong mechanism where hydride from the flavin first attacks the C_β atom and a proton from the solvent is subsequently added onto the C_α atom. The activating group on the C_α atom allows the polarization of the C=C bond, necessary for hydride addition. Overall, this trans-addition is highly stereoselective and provides enantiomeric excesses of often >99% (Figure 7).[44] These biocatalysts are attractive tools for industrial applications because of their exquisite stereoselectivity but also their broad acceptance of various activating groups, enlarging the scope of chiral products obtained. The OYE family accepts a wide range of activating groups, such as ketone, carboxylic acid, carboxylic ester, lactone, imide, nitrile, nitro, and aldehyde. Ynones are also reduced, first to enones and ultimately to α,β-saturated ketones.[45] The diversity of activating groups accepted by OYEs has led to the development of a remarkable range of chiral compounds that could potentially be used as building blocks in the synthesis of larger molecules. Comprehensive reviews have been published recently.[46,47]

A series of α,β-unsaturated nitriles were converted to the corresponding nitrile products in high yield and optical purity.[48] This class of compounds is of great value for synthetic applications as they can be easily converted to carboxylic acids, amides or aldehydes. Various *Z*-configured para-substituted phenyl butenenitriles as well as one more demanding pharmaceutical building block (6-chloro-5-methylspiro[1*H*-indene-1,4′-piperidine]-3-carbonitrile) were converted with good to excellent yields and perfect stereoselectivity furnishing the corresponding (*R*)-products in *ee* up to 99% (absolute configuration of pharmaceutical

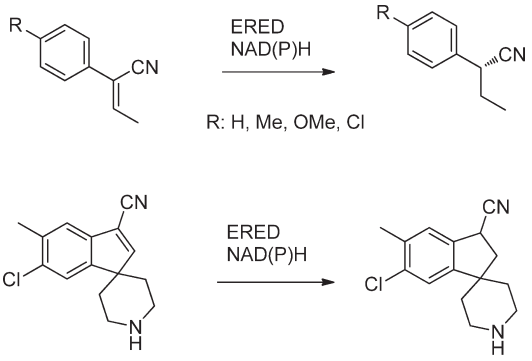


Figure 8. Bioreduction of nitriles using enoate reductases (ERED). Adapted from ref 48 with permission. Copyright 2008 Elsevier.

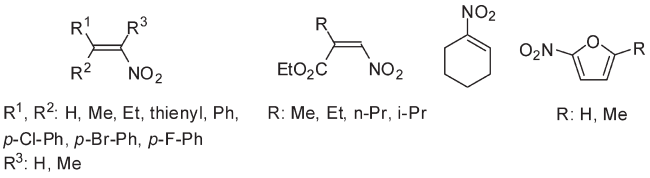


Figure 9. Nitroalkenes converted by OYE homologues.

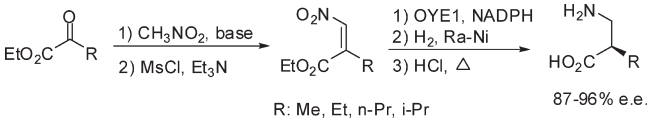


Figure 10. Chemo-enzymatic route to β^2-amino acids with reduction of β-nitroacrylate with OYE. Adapted with permission from ref 55. Copyright 2006 American Chemical Society.

synthon not determined) using a set of commercially available ene-reductases (Figure 8).

Equally important synthetic intermediates are nitro compounds as they can be further transformed to amines, carbonyl compounds or hydrocarbons.[49] Examples abound where nitro olefins are converted to their saturated nitro analogues using whole cell catalysts or isolated enzymes. YqjM, OPR1, OPR3, OYE1−3, NCR, PETN reductase, NEM reductase, morphinone reductase, TOYE, XenA, and EBP1 showed various reactivity and stereoselectivity patterns in the reduction of a range of nitroalkenes (Figure 9).[47,50−55] β-Nitroacrylates were shown to be good substrates for OYE1 and the bioreduction step was incorporated into a multistep synthetic route to β^2-amino acids starting from α-keto esters.[55] After the enzymatic reduction, hydrogenation of the nitro group to the amine with Raney-Ni followed by acid-catalyzed deprotection of the ester yielded β^2-amino acids in high yield and optical purity (91−96% *ee*), except in the case of 3-alkyl-substituted products, which were racemic (Figure 10).

Interestingly, the bioreduction of *sec*-nitrocompounds furnished the corresponding carbonyl compounds via a reaction presented as a biocatalytic equivalent to the Nef reaction. The reduction of (*E*)-1-nitro-2-phenylpropene was shown to proceed sequentially to the nitroalkane first, then further reduced to the corresponding oxime, which was finally converted to 2-phenylpropanal. Different ratios of the products were obtained depending on the enzyme used in the reduction.[56]

dx.doi.org/10.1021/cr200013n |*Chem. Rev.* 2011, 111, 4088–4110

Optically active butanolides, including chiral 2,3-disubstituted butanolides and 4-substituted butanolides, were produced after reduction of 2-butenolides using two stereocomplementary reductases from cultures cells of *Glycine max*, p51 and p83. The two enzymes were used efficiently in the reductive resolution of 4-substituted 2-butenolides to furnish chiral 4-substituted butanolides.[57] *O*-Protected acyloins could be obtained through the bioreduction of α,β-unsaturated alkoxy ketones. α-Alkoxy enones were converted by a large set of OYEs while β-alkoxy enones were not.[58] Furan derivatives are also substrates to OYEs but have not been investigated in regards of product purity and absolute configuration.[50,59]

While the reaction catalyzed by OYEs is stereoselective in all cases, variability (in final *ee* or stereopreference) is observed throughout the whole family and has allowed the development of enzyme- and substrate-based stereocontrols. In the first case, chemists have the option to synthesize both enantiomers of the product in pure form by choosing the biocatalyst displaying the desired stereoselectivity. Thus starting from the same substrate, either the (*R*)- or (*S*)-enantiomer is accessible. Indeed, several OYEs have been found to be stereocomplementary (enzyme-based stereocontrol, Figure 11), and one striking example was given by OPR homologues from *Lycopersicon esculentum* (tomato). While OPR1 catalyzed the (*R*)-stereoselective reduction of 1-nitro-2-phenylpropene furnishing the corresponding (*R*)-1-nitro-2-phenylpropane in optically pure form, OPR3 yielded the (*S*)-1-nitro-2-phenylpropane with high *ee* value. In both cases, the reaction almost reached completion. This striking difference was suggested to be based on a subtle change in amino acid sequence, where a lysine residue (Lys-79) in OPR1 was substituted with a proline in OPR3 (Pro-75), thus inducing a conformational change that forced the substrate to bind in the opposite orientation and led to the formation of the opposite enantiomer.[60,61] In substrate-based stereocontrol, the initial configuration of the substrate dictates the stereochemical outcome of the reaction (Figure 12). The *R*/*S*- configuration of the reduced products were shown to depend on the *E*/*Z*-configuration of the original substrates, as observed in the reduction of methyl 2-chloro-2-alkenoates by fermentation with baker's yeast (the actual substrates were the corresponding acid forms obtained after hydrolysis of the esters by unspecific esterases).[62] The same effect was observed in the reduction of citral with OYE1.[63] While examples abound in the literature, they usually give rise to different products. For instance, by varying the ring-size of cycloalkenones such as 2-methylcyclopentenone and 2-methylcyclohexenone, both NCR from *Zymomonas mobilis* and OYE3 showed opposite stereopreference and yielded (*S*)-2-methylcyclopentanone and (*R*)-2-methylcyclohexanone, respectively.[64] Variations of the substituent in α-alkoxy enones also led to stereodivergent selectivity, where increasing the size of the *O*-protecting group switched the acyloin product configuration from (*S*) to (*R*).[58] The reduction of citraconic (*Z*-) and mesaconic (*E*-) acid dimethylesters however led to strictly opposite enantiomers upon reduction employing YqjM. Both (*R*)- and (*S*)-dimethyl 2-methylsuccinate were obtained individually and with high *ee* (>99%).[65] On the contrary, the reduction of (*E*)- and (*Z*)-isomers of various nitro olefins proceeded in a stereoconvergent manner using PETN reductase from *Enterobacter cloacae* PB2.[54]

Recently, members of the OYE family were applied to the chemoenzymatic preparation of non racemic α-methyl dihydrocinnamaldehyde derivatives employed as olfactory principles in perfumes (Lilial, Helional).[66] Given that the stereochemistry of most aldehydes used as fragrances and flavors influences the

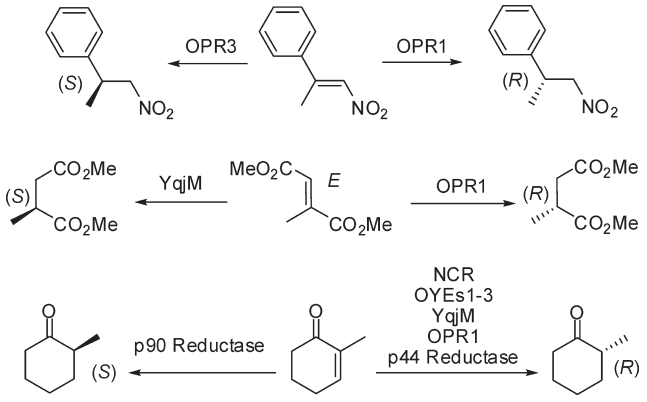


Figure 11. Enzyme-based stereocontrol with OYE homologues. Reprinted from ref 46. Copyright 2010 John Wiley & Sons, Inc. Reprinted with permission of John Wiley & Sons, Inc.

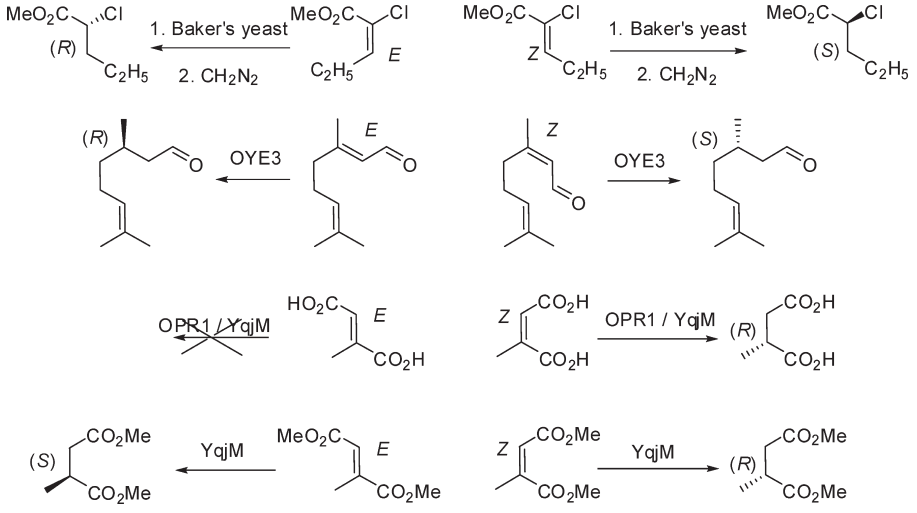


Figure 12. Substrate-based stereocontrol with OYE homologues. Reprinted from ref 46. Copyright 2010 John Wiley & Sons, Inc. Reprinted with permission of John Wiley & Sons, Inc.

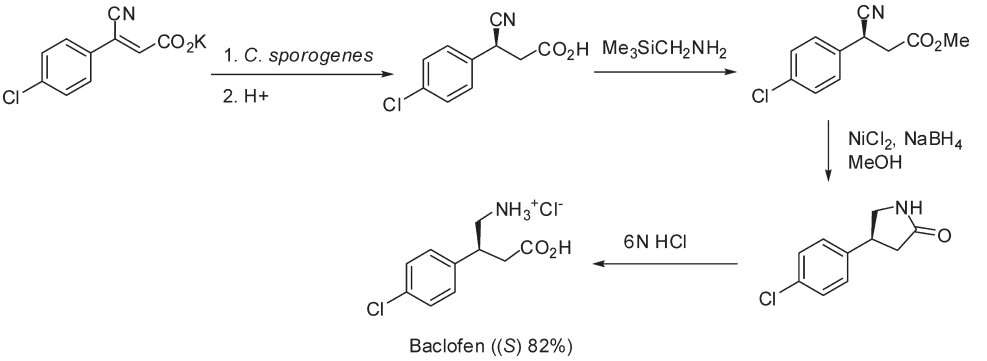


Figure 13. Chemoenzymatic route to baclofen including *Clostridium sporogenes*-catalyzed reduction step. Adapted from ref 78. Reproduced by permission of the Royal Society of Chemistry.

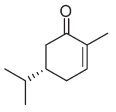
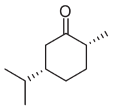
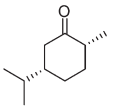
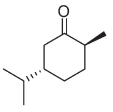
ultimate odor of the compound,[67−69] advantage was taken of the stereocomplementary behavior of some OYE homologues to furnish either (*R*)- or (*S*)-configurated aldehydes. Though OPR1 and YqjM selectivities toward the (*R*)-enantiomer were modest (max. 53%), the (*S*)-enantiomer was obtained in almost perfect optical purity (97%) with NCR from *Zymomonas mobilis* and OYEs 1−3 in an aqueous−organic biphasic system containing 20% (v/v) *t*-butyl methyl ether. This biocatalytic method was shown to be a competitive alternative to other asymmetric hydrogenation protocols. Another industrially relevant example was reported with the synthesis of the "Roche ester" ((*R*)-3-hydroxy-2-methylpropanoate), a popular chiral building block for the synthesis of vitamins (e.g., α-tocopherol), fragrance components (e.g., muscone), and antibiotics (e.g., calcimycin, palinurin, rapamycin, 13-deoxytedanolide or dictyostatin) and natural products (e.g., spiculoic acid A). Using a set of ene reductases, the Roche ester, as well as several derivatives bearing different protective groups on the hydroxyl moiety, could be obtained in high optical purity. Higher conversion levels were obtained with an allyl or benzyl ether group (preventing strong hydration of the allylic alcohol).[70]

Enoate Reductases from Anaerobes. Enoate reductases (EC. 1.3.1.31) initially characterized by Simon et al. and capable of catalyzing the stereoselective reduction of α,β-unsaturated carboxylate anions were discovered and isolated from anaerobes such as *Clostridium* spp. (*Clostridium kluyveri, C. tyrobutyricum, and C. sporogenes*).[71,72] These enzymes are not part of the OYE family of flavoproteins, they are iron−sulfur NADH dependent flavoproteins and have been shown to catalyze the reduction of carbon−carbon double bonds of (weakly activated) 2-enoates as well as of α,β-unsaturated aldehydes, cyclic ketones, methylketones, and recently, β,β-disubstituted aromatic nitroalkenes in a stereoselective manner.[73−77] β-Aryl-γ-amino acids were obtained from a chemoenzymatic route incorporating the bioreduction of β-aryl-β-cyano-α,β-unsaturated carboxylic acids using cell extract of *Clostridium sporogenes*. This strategy was successfully applied to the synthesis of baclofen (β-(4′-chlorophenyl)-γ-GABA), a chiral pharmaceutical ingredient (Figure 13).[78]

Protein Engineering. In contrast to the situation with alcohol dehydrogenases, the use of protein engineering to improve the overall efficiency (mostly stereoselectivity) of OYE-like enzymes has only been recently implemented for synthesis applications and shows already great promises (Table 2).

While mutational studies were already common techniques to investigate the active site residues of OYEs,[79−82] the first application of protein engineering for enhanced biocatalytic properties was shown with OYE1. Stewart et al. mutated one single residue that was identified through modeling of the Michaelis complex with the (poor) substrate 3-ethyl-2-cyclohexenone. Trp116 was indeed likely to interact with the β-substituent of the substrate and site-saturation mutagenesis was used to create mutation at that position. 200 colonies were screened for catalytic activity but all activities turned out lower than wild type activity (with similar stereoselectivity), while striking differences were obtained with carvone-like substrates. W116I induced a flipped binding mode of the substrates resulting in a different stereo-outcome of the reaction, still in high enantiomeric excess, thus providing stereocomplementary enzymes for the reduction of these specific molecules.[83] Reetz et al. showed shortly after that only minor changes were required to completely alter YqjM stereoselectivity or activity but more important, improve tolerance to new substrates and switch the stereopreference for various α,β-unsaturated carbonyl compounds. A large number of variants were investigated, employing an iterative saturation mutagenesis (ISM).

Table 2. Application of Protein Engineering to OYE Homologues[83,84]

Substrate	Product		
	OYE1 WT	OYE1 W116F	OYE1 W116I
			
Configuration	*cis*-(1*R*,4*S*)	*cis*-(1*R*,4*S*)	*trans*-(1*S*,4*S*)
Conversion (%)	59	79	>98
d.e. (%)	> 98	89	>98
	YqjM WT	YqjM C26W/A104Y	YqjM C26G/A60V
			
Configuration	(*R*)	(*R*)	(*S*)
Conversion (%)	3	72	78
e.e. (%)	75	98	>99

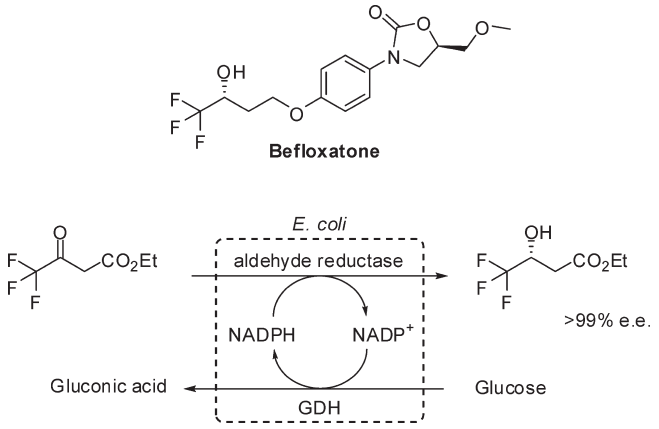


Figure 14. Lonza process for the biocatalytic preparation of a building block for befloxatone synthesis. Adapted from ref 85. Copyright 2003 Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

Here also, only a limited number of residues were necessary to induce switch of stereopreference on 3-alkyl-substituted 2-cyclohexen-1-ones and 2-cyclopenten-1-ones, alter or improve the product enantiopurity (only two residues necessary for instance with 3-methylcyclohexenone).[84] Both works have in common the use of structure-guided approaches applied to directed evolution and show that reducing steric hindrance around bulky substrates can lead to stereoswitches while mutations in a fairly confined environment around the binding pocket can improve or reverse the stereopreference on specific substrates.

This strengthens further the available biocatalytic toolbox for the reduction of carbon−carbon double bonds using this already versatile class of enzymes.

2.1.3.3. Other Reductases. Aldehyde Reductases. Lonza has developed a biocatalytic process for the synthesis of (*R*)-ethyl 4,4,4-trifluoro-3-hydroxybutanoate, a building block of befloxatone, an antidepressant monoamine oxidase-A inhibitor. The main step is the reduction of ethyl 4,4,4-trifluoroacetoacetate using an aldehyde reductase from *Sporobolomyces salmonicolor* coexpressed in *E. coli* cells with a glucose dehydrogenase for cofactor regeneration. This process is carried out in a biphasic system to prevent substrate and product inhibition of the reductase and allows the formation of the product in >99% *ee* and 50% yield (Figure 14).[85]

Diketoreductases. The stereoselective reduction of two carbonyl groups in the same molecule to form a chiral *β*, *δ*-dihydroxy product by a single enzyme is of great synthetic interest as it produces two stereogenic centers in one-pot reaction. Several reports showed that such reaction can be catalyzed by one ketoreductase from *Acinetobacter* spp. This enzyme, named ketoreductase III by Patel et al., stereoselectively reduced 3,5-dioxo-6-(benzyloxy)hexanoic acid ethyl ester to the corresponding syn-(3*R*,5*S*)-dihydroxy ester. The different reaction rates for the (3*R*)- and (5*S*)-hydroxy esters suggested that reduction of the two carbonyl groups was likely to be due to sequential reactions by the same enzyme. Cells or extracts of recombinant *E. coli* efficiently reduced the diketoester to the corresponding *syn*-diol in 99.3% yield, 100% *ee*, and 99.8% de.[86−88]

Shortly after, Chen et al. reported the cloning, expression and characterization of a diketoreductase (DKR) from another *Acinetobacter* sp. that was highly stereoselective in the reduction of both carbonyl groups in a *β*,*δ*-diketo ester.[89,90] The

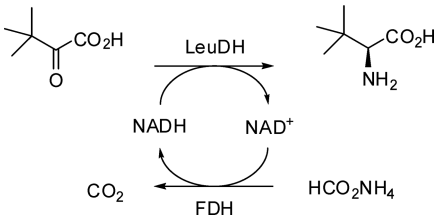


Figure 15. LeuDH-catalyzed reductive amination of trimethyl pyruvic acid to *tert*-leucine using FDH as cofactor regeneration system. Adapted from ref 92, Copyright 1995, with permission from Elsevier.

recombinant enzyme was combined with a cofactor regenerating system for the synthesis of statin side chains.

Amino acid dehydrogenases. Amino acid dehydrogenases (DH) constitute a powerful tool for the production of enantiomerically pure amino acids through the reductive amination of α-ketoesters or acids. Unnatural amino acids such as optically pure amino acids with bulky side chains are of great value as synthons in the pharmaceutical industry, and biocatalytic routes have been developed to allow their synthesis. A wide range of possible substrates with bulky hydrophobic side chains for instance was synthesized and converted by leucine dehydrogenase (LeuDH) and phenylalanine dehydrogenase (PheDH). Both LeuDH and PheDH present broad substrate specificity. While LeuDH accepts 2-oxo acids with hydrophobic, aliphatic, branched and unbranched carbon chains (up to a straight chain of eight C-atoms) and some alicyclic keto acids as substrates, PheDH additionally accepts aromatic substrates.[91−93] Since equilibrium is in favor of the amino acid product, conversion of the α-ketoacid is quantitative and maximum conversion often approaches 100% (at high substrate concentrations however, various limiting factors need to be taken into consideration[94]). Coupling the amino acid dehydrogenase with a cofactor recycling system such as formate dehydrogenase (FDH)/formate (ammonium formate also used as source of ammonium for the reductive amination) allowed large-scale industrial production of L-amino acids such as *tert*-leucine (Figure 15). The continuous reduction of trimethyl pyruvate to L-*tert*-leucine has also been carried out in an enzyme-membrane reactor (EMR),[95] while other approaches include the use of whole-cell biocatalysts.[25,96,97] High yields (>70%) and productivities (>600 $g \cdot L^{-1}.d^{-1}$) were reported.[98]

Another interesting application of amino acid dehydrogenases concerns the preparation of (*S*)-3-hydroxyadamantylglycine from 2-(3-hydroxy-1-adamantyl)-2-oxoethanoic acid using a modified form of a recombinant PheDH cloned from *Thermoactinomyces intermedius* and expressed in *Pichia pastoris* or *Escherichia coli*. The nonproteinogenic amino acid is a key intermediate required for the synthesis of Saxagliptin, a dipeptidyl peptidase IV inhibitor under development for treatment of type 2 diabetes mellitus (Figure 16). *E. coli* coexpressing PheDH and FDH from *Pichia pastoris* for cofactor regeneration allowed the successful reductive amination of the ketoacid in several kg scale (40 kg in 800-L vessel and 99% conversion).[99]

L-6-Hydroxynorleucine, a chiral intermediate required in the synthesis of omapatrilat (Vanlev), an antihypertensive drug, was obtained by deracemization of racemic 6-hydroxynorleucine via treatment with D-amino acid oxidase and catalase (either porcine kidney D-amino acid oxidase and beef liver catalase or *Trigonopsis variabilis* whole cells). In the second step (after obtaining unreacted enantiopure L-6-hydroxynorleucine), the reductive

amination procedure was used to convert the mixture containing 2-keto-6-hydroxy hexanoic acid 3 and L-6-hydroxynorleucine entirely to L-6-hydroxynorleucine (>98% *ee*) with yields of 97% from racemic 6-hydroxynorleucine (starting at 100 $g \cdot L^{-1}$, Figure 17).[100,101]

Last, deracemization of racemic mandelic acid to optically pure non-natural L-phenylglycine was obtained via a redox-neutral biocatalytic cascade using three enzymes. Optically pure L-phenylglycine was obtained in >97% *ee* and 94% conversion without the requirement of any additional redox reagents in stoichiometric amounts. While the enantiomers of racemic α-hydroxy acid were interconverted with mandelate racemase, D-mandelic acid was oxidized by a D-selective mandelate dehydrogenase to give the corresponding α-oxo acid. The latter was concurrently transformed to the corresponding α-amino acid via an asymmetric reductive amination using L-selective amino acid dehydrogenase. Since the formal hydrogen abstracted in the oxidation was used in the reduction, both reactions (the enantioselective oxidation and the stereoselective reduction) were run simultaneously.[102]

Highly interesting was the recent report of a broad substrate range, nicotinamide cofactor dependent, and highly stereoselective D-amino acid dehydrogenase, that was created using both rational and random mutagenesis performed on the enzyme meso-diaminopimelate D-dehydrogenase. Since D-amino acid dehydrogenases are not ubiquitous in nature as opposed to their L-counterpart, this opens up new possibilities for the synthesis of D-amino acids. The mutant was capable of producing D-amino acids via the reductive amination of the corresponding 2-keto acids with ammonia. The mutant for instance catalyzed the amination of cyclohexylpyruvate with ammonia to produce D-cyclohexylalanine. The cofactor, NADPH, was recycled using glucose and glucose dehydrogenase. The glucose oxidation product, gluconolactone, spontaneously hydrolyzed irreversibly to gluconic acid driving the reaction to completion (Figure 18).[103]

Aldo-keto Reductases. Two aldo-ketoreductases (AKR) were found to catalyze the reduction of prochiral α-ketocarbonyl substrates to chiral α-hydroxy carbonyl products, which are commonly encountered chiral auxiliaries and synthons for the asymmetric synthesis of natural products, including antitumor agents, antibiotics, pheromones, and sugars. The enzymes, strictly NADPH-dependent, displayed high regioselectivity and broad substrate specificities and reduced α-ketocarbonyls at the keto moiety most proximal to the terminus of the alkyl chain to produce α-hydroxy carbonyls. Gox0644 and Gox1615 were stereocomplementary in the reduction of 2,3-pentanedione and produced 2-(*R*)-hydroxy-pentane-3-one and 2-(*S*)-hydroxy-pentane-3-one, respectively. Both enzymes also reduced 1-phenyl-1,2-propanedione to 2-hydroxy-1-phenylpropane-1-one, which is a key intermediate in the production of numerous pharmaceuticals,

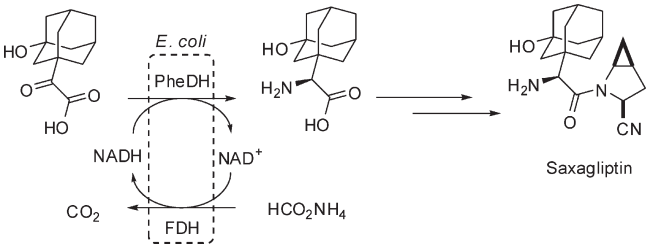


Figure 16. Reductive amination to (*S*)-3-hydroxyadamantylglycine, a precursor to saxagliptin, using a PheDH/FDH system. Adapted from ref 99. Copyright 2007 Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

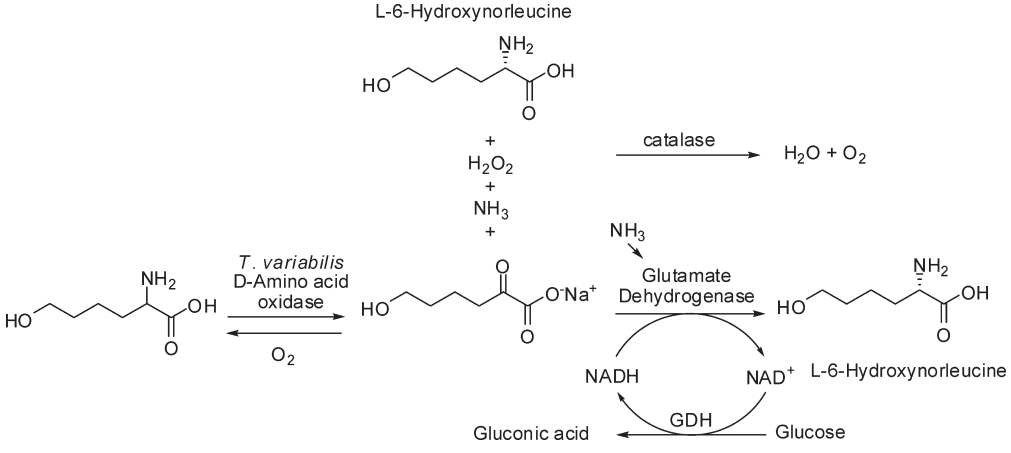


Figure 17. Deracemization of 6-hydroxynorleucine and reductive amination to enantiopure L-6-hydroxynorleucine. Adapted from refs 100 and 101, Copyright 1999 and 2001, with permission from Elsevier.

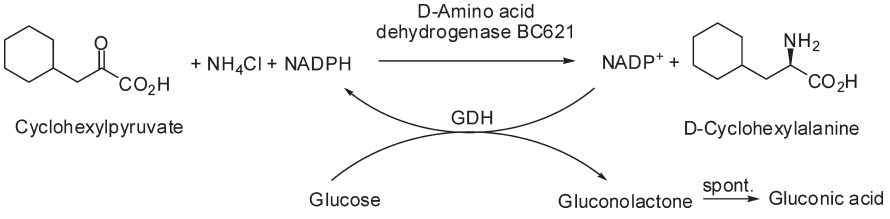


Figure 18. Reductive amination of cyclohexylpyruvate using an engineered meso-diaminopimelate D-dehydrogenase and a GDH recycling system. Adapted with permission from ref 103. Copyright 2006 American Chemical Society.

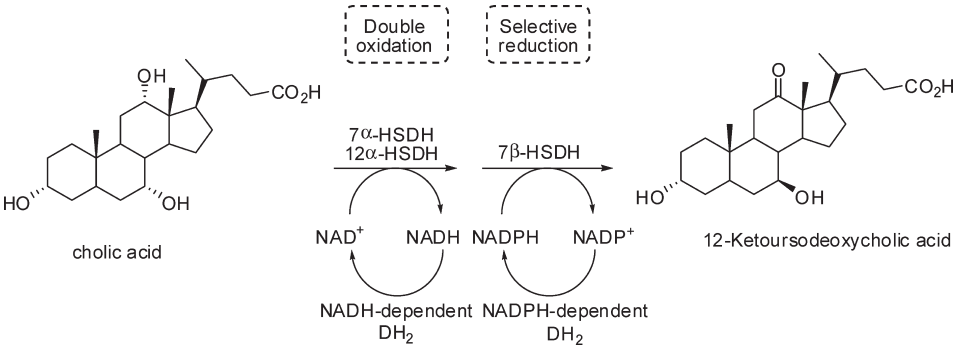


Figure 19. One-pot multienzymatic synthesis of 12-ketoursodeoxycholic acid ruled by cofactor specificity of five biocatalysts. Adapted with permission from ref 105. Copyright 2009 Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

such as antifungal azoles and antidepressants. While Gox0644 displayed highest activities with 2,3-diones, α-ketoaldehydes, α-keto esters, and 2,5-diketogluconate, Gox1615 displayed a broader substrate spectrum reducing a variety of α-diketones and aldehydes.[104]

2.1.4. Outlook. Recently, an interesting one-pot/five-enzyme system has been implemented for the synthesis of 12-ketoursodeoxycholic acid, a key intermediate for the synthesis of the drug ursodeoxycholic acid, from cholic acid. The reaction scheme relied on hydroxysteroid dehydrogenases (HSDH) displaying different cofactor preferences (Figure 19). Cholic acid was first oxidized to 3α-hydroxy-7,12-dioxo-5β-cholanoic acid by NADH-dependent 7α-HSDH and 12α-HSDH, while the product was regio- and stereoselectively reduced by an NADPH-dependent 7β-HSDH. Coupled in situ regeneration systems (NADH-dependent lactate dehydrogenase LDH and NADPH-dependent glucose dehydrogenase GDH) were exploited not only to allow the use of catalytic amounts of the cofactors, but also to provide the necessary driving force to opposite reactions (i.e., oxidation and reduction) acting on different sites of the substrate molecule. Undesired outcomes of effective reaction equilibria were attributed to nonperfect cofactor specificity of GDH, thus leading to nonspecific bioconversion process. This was overcome by compartmentalizing the oxidative and reductive catalysts.[105]

2.2. Oxidation

Oxidation reactions involved in the production of pure enantiomers include deracemization of a racemic mixture, asymmetrization of meso diols or desymmetrization from a prochiral compound.

2.2.1. Deracemization. Deracemization reaction traditionally differs from stereoinversion (1.1.1.) due to the use of a chemical reactant to perform the reduction reaction. It consists of a cyclic selective enzyme-catalyzed oxidation/nonselective chemical reduction process (also called cyclic deracemization[106,107]). A highly enantioselective oxidase oxidizes one enantiomer of the racemic substrate into a prochiral product that is subsequently reduced back to the racemic starting material, thus enriching the starting material with the unreactive enantiomer. After a limited amount of cycles (provided the enzyme displays a high enantioselectivity), enantiomeric excess can exceed 99%.

Optically pure unnatural amino acids for instance can be obtained via the concerted use of a D-amino acid oxidase yielding a mixture of the corresponding imine and the unreacted amino acid enantiomer, and a chemical reductant such as $NaBH_4$, $NaCNBH_3$, amine-borane complex or Pd/C-ammonium formate for the non selective reduction of the imine to the racemic amino acid. L-Amino acid oxidase was also successfully employed so that both enantiomeric forms of an amino acid could be obtained.[108,109] Several β- and γ-substituted-α-amino acids were also deracemized using L- or D-amino acid oxidase and sodium cyanoborohydride or sodium borohydride (Figure 20).[110]

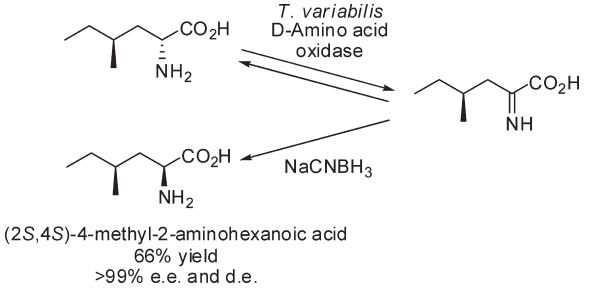


Figure 20. Deracemization of 4-methyl-2-aminohexanoic acid using D-amino acid oxidase and sodium cyanoborohydride. Adapted from ref 110. Reproduced by permission of the Royal Society of Chemistry.

Unselective electrochemical reduction was also combined to a selective enzymatic oxidation for the deracemization, stereoinversion and asymmetric synthesis of L-leucine (starting from racemic leucine, D-leucine or 4-methyl-2-oxovaleric acid) in a batch reactor (Figure 21). D-Amino acid oxidase (D-AAO) from *Trigonopsis variabilis* was used as enzyme and the reaction was conducted in a batch reactor with a graphite electrode at −1.5 V vs Ag/AgCl in $(NH_4)_2SO_4/NH_3$ buffer (pH 10). The electroenzymatic synthesis yielded 3.5 $mmol \cdot L^{-1} \cdot d^{-1}$ of L-leucine (*ee* 91%).[111]

Other deracemization methods for the preparation of enantiopure non-natural α-amino acids have been reviewed.[112]

2.2.2. Asymmetrization of Meso Diols. Horse liver alcohol dehydrogenase (HLADH) regio- and stereoselectively oxidizes one of the hydroxy groups in a polyhydroxylated molecule[113,114] and for that reason has been successfully employed in the asymmetrization of *meso*-diols to produce enantiomerically pure γ- and δ-lactones.[115−117] In general, HLADH accepts primary and secondary racemic alcohols as well as prostereogenic mono- and dihydroxy compounds. Ideal substrates are cis *meso*-1,4- and *meso*-1,5-diols, which are attacked at the pro-*S* hydroxymethyl group providing hydroxy-aldehydes which immediately cyclize to hemiacetals followed by further oxidation to the stable (1*S*)-γ- and (1*S*)-δ-lactones (Figure 22).[118,119]

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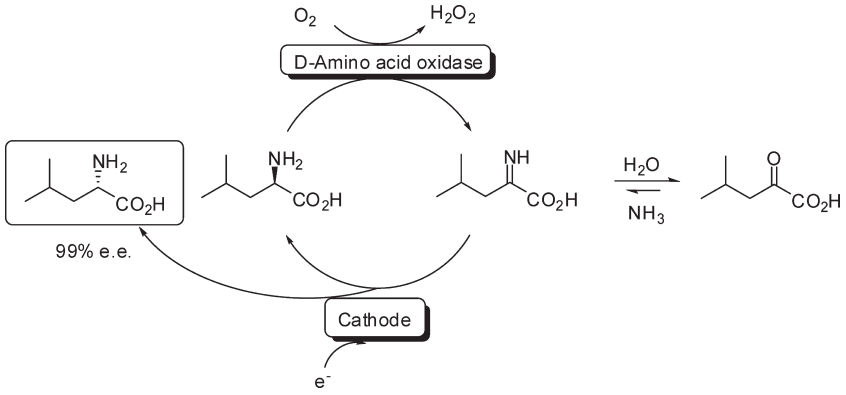

Figure 21. Electroenzymatic cyclic deracemization of leucine. Reprinted from ref 111, Copyright 2008, with permission from Elsevier.

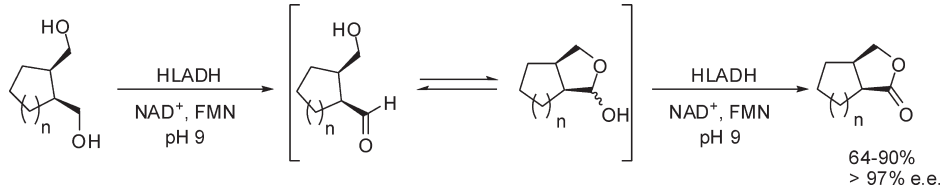

Figure 22. Asymmetrization of meso diols through HLADH-catalyzed oxidation to lactones. Adapted from ref 118. Copyright 1997 Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

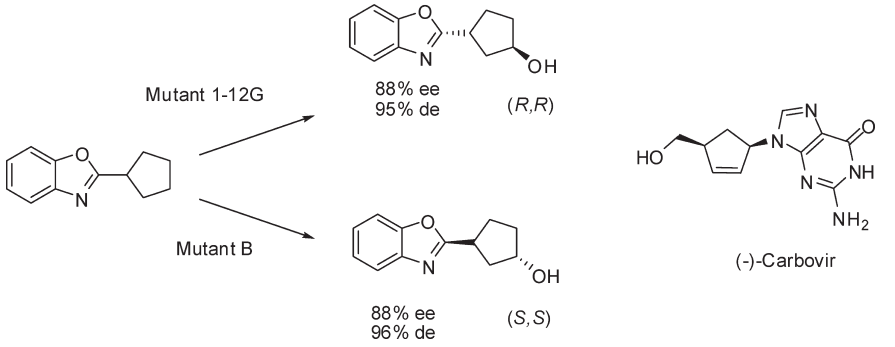

Figure 23. Application of cytochrome P450 monooxygenase mutants for the synthesis of potential precursors to Carbovir. Adapted from ref 132. Reproduced by permission of the Royal Society of Chemistry.

The synthesis of optically pure or enantiomerically enriched lactones via the oxidation of nonsymmetric acyclic racemic 1,5 and 1,6-diols with HLADH was recently reported. Regioselective oxidation of primary-secondary 1,5-diols and 1,6-diols afforded enantiomerically enriched δ-lactones and ε-lactones (in high enantiomeric excess 85−99%).[120]

2.2.3. Desymmetrization. Asymmetric synthesis by desymmetrization using oxidative reactions relies on the direct incorporation of molecular oxygen into a non chiral substrate using (mono- and di-) oxygenases.

2.2.3.1. Monooxygenases. Cytochrome P450 Monooxygenases. Cytochrome P450 monooxygenases (CYPs) constitute a family of heme-containing enzymes displaying extremely diverse catalytic activities. These versatile and powerful biocatalysts have indeed been shown to catalyze carbon hydroxylation, epoxidation, heteroatom dealkylation and oxidation, phenolic couplings and reductive denitrations, desaturations, and oxidative ester cleavages or aldehyde scissions.[121,122] Recent literature gives a comprehensive survey on this class of enzymes.[123−127]

The use of CYPs in asymmetric synthesis has been greatly improved by use of protein engineering. The enzyme BM-3 from *Bacillus megaterium*, for instance, has been engineered for the regio- and enantioselective alkane hydroxylation and alkene epoxidation.[128−131] These mutants also proved active in the hydroxylation of 2-cyclopentylbenzoxazole, yielding a compound, which, once deprotected, could be used as starting material in the synthesis of Carbovir, a carbocyclic nucleoside potentially active against human HIV (Figure 23). Depending on the mutant employed, variation in stereoselectivity was observed and both (*S*,*S*) and (*R*,*R*)-enantiomers could be obtained in high *ee* and de (up to 88% and 96%, respectively). The wild-type in comparison was poorly active and barely selective.[132] Similarly, other mutants displayed selective hydroxylation activities on various 2-arylacetic acid esters, yielding the corresponding esters of (*S*)-mandelic acid in up to 88% yield and 93% *ee*. (*R*)-6-Hydroxybuspirone was also obtained through the highly selective hydroxylation of buspirone (>99.5% *ee*), both compounds being antianxiety agents.[133]

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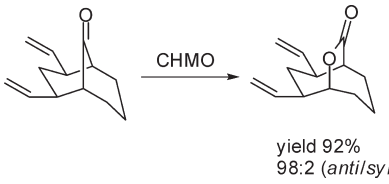

Figure 24. Example of demanding substrate accepted by CHMO from *Xanthobacter sp. ZL5*. Adapted from ref 143. Copyright 2008 Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

CYPs (both human and bacterial recombinant forms) are also becoming competitive in other areas such as drug metabolites synthesis, an important aspect in pharmaceuticals development.[134] Directed evolution was used to generate variants of P450 BM3 heme domain peroxygenase from *Bacillus megaterium* that showed enhanced production of authentic human metabolites of propanolol. In a first round, the mutations specifically targeted the active site residues, while the second library was constructed by applying error-prone PCR to the most active first mutants. The final mutant contained five amino acid substitutions compared to the parent (a mutant of the wild-type generated in a previous study) and showed higher production of the major metabolites.[135]

Baeyer−Villiger Monooxygenases. Synthetic interest in Baeyer−Villiger monooxygenases (BVMOs) was first raised with cyclohexanone monooxygenase[136] and since then many BVMOs have been extensively used for the stereoselective oxidation of cyclic and linear ketones to produce optically pure lactones and esters, respectively, even on kilogram scale.[137−140] BVMOs (EC 1.14.13.*x*) are exquisitely regio, stereo-, and enantioselective flavin-dependent enzymes that display a broad substrate spectrum. Catalysis is achieved by formation of a peroxy catalyst (a peroxyflavin) upon reaction of the flavin with NADPH and molecular oxygen after which the peroxyflavin enzyme reacts with suitable substrates (oxygenation).[141,142]

BVMO from *Xanthobacter* sp. ZL5 was used in the biotransformation of various prochiral substrates. This enzyme catalyzed the desymmetrization of cyclic ketones bearing different chemical features. Cyclohexanone monooxygenase (CHMO) in particular was able to accept and oxidize sterically demanding substrates with excellent enantioselectivity. Six substrates gave access to highly interesting enantiopure lactones containing sterically hindered structural motifs (Figure 24). All lactones were obtained with excellent enantiomeric excesses.[143]

The regio- and stereoselective allylic biohydroxylation of D-limonene to (+)-*trans*-carveol (valuable fragrance and flavor compound) has been reported using resting cells of *Cellulosimicrobium cellulans* EB-8−4. High conversion and high yield along with almost perfect regio- and stereoselectivity (>99%) were obtained in the biohydroxylation of 11.6 mM of D-limonene in a closed shaking flask, giving 10 mM of (+)-*trans*-carveol, and 0.30 mM of carvone as the only by-product. The reaction is most likely catalyzed by a BVMO.[144]

Phenylacetone monoxygenase (PAMO) has a limited substrate scope as it accepts only phenylacetone and related linear phenyl-substituted derivatives.[145,146] Reetz et al. have circumvented this drawback by applying directed evolution to PAMO. Domain movements were induced via allostery, leading to a different shape of the binding domain. One double mutant was found to be active on a set of 2-substituted cyclohexanone derivatives, usually not accepted by the wild-type, and was used in the oxidative kinetic resolution yielding the corresponding lactone with high enantioselectivity. 4-Substituted cyclohexanone derivatives were also converted to the enantiopure lactone, and the double mutant was still active on phenylacetone.[147,148]

For ketones that do not react in an enantioselective manner with BVMOs, directed evolution can be employed, and has been successfully used for the desymmetrization of 4-hydroxycyclohexanone with CHMO.[149]

One recent study showed that the use of BVMO to gain access to β-amino acids and β-amino alcohols is possible. In this new enzymatic route to enantiopure β-amino acids, *N*-protected β-amino ketones served as racemic substrates in a kinetic resolution, leading to an enrichment of *N*-protected β-amino esters. Hydrolysis in a second step furnished pure *N*-protected β-amino acids. Interestingly, insertion of oxygen was observed on both side of the carbonyl moiety, which resulted in the production of "abnormal" and "normal" lactones, which upon hydrolysis of the ester group yielded β-amino acids and β-amino alcohols, respectively. Among the various BVMO investigated, most enzymes (cyclododecanone monooxygenase CDMO and cyclohexanone monooyxygenases CHMO from various sources) showed high enantioselectivity (*ee* > 99% for both products) and were found to be enantiocomplementary, allowing production of both enantiomeric forms of the amino acid. *N*-Protected 5-amino-3-one for instance was converted to both Baeyer−Villiger esters using whole cells of *E. coli* expressing CDMO, and these products, once isolated and purified, were further hydrolyzed using *Candida antarctica* lipase B (CAL-B) to furnish the *N*-protected β-amino acid and *N*-protected β-amino 4-methyl-1-pentanol (Figure 25). Nonprotected β-amino ketones were interestingly not converted by BVMO.[150]

Monooxygenases can also be used to produce chiral sulfoxides and research in this field has been recently reviewed.[151] Their classification, mode of action, and various biotechnological aspects have been reviewed, while excellent surveys on synthetic applications are available.[124,152]

2.2.3.2. Dioxygenases. Dioxygenases are powerful biocatalysts that have been used in various enzymatic dihydroxylation reactions of aromatics for enantioselective synthesis, including toluene, naphthalene, or biphenyl dioxygenases.[153]

Toluene dioxygenases (TOD) catalyze the oxidation of aromatic compounds to furnish the corresponding *cis*-cyclohexadiene diols and present great synthetic potential as there is currently no synthetic equivalent. The stereochemistry of TOD-catalyzed oxidation of 1,4-disubstituted benzene compounds has been shown to be preferably *cis*-, thus generating the corresponding *cis*-dihydrodiols.[154,155] Dibromobenzenes (*o*-, *m*-, and *p*-isomers) have been converted to their corresponding *cis*-cyclohexadiene diols by whole-cell fermentation with *E. coli* cells overexpressing TOD.[156] *m*-Dibromobenzene for instance was used to produce, as single isomer, a starting material for the synthesis of the Amaryllidaceae constituent narciclasine (yield of 4 $g \cdot L^{-1}$).[157−159] TOD was also used in the asymmetric total synthesis of (+)-pancratistatin and (+)-7-deoxypancratistatin (promising antitumor agents), where bromobenzene was transformed to (1*S*,2*S*)-3-bromocyclohexa-3,5-diene-1,2-diol using recombinant TOD expressed in *E. coli*.[160]

TOD-catalyzed *cis*-dihydroxylation of phenols has even led to the discovery of new enantiopure cyclohexenone *cis*-diol (as well as *o*-quinol dimer and phenol hydrate) metabolites with synthetic potential. *p*-Xylenol for instance was converted to the corresponding cyclohexenone *cis*-diol.[161]

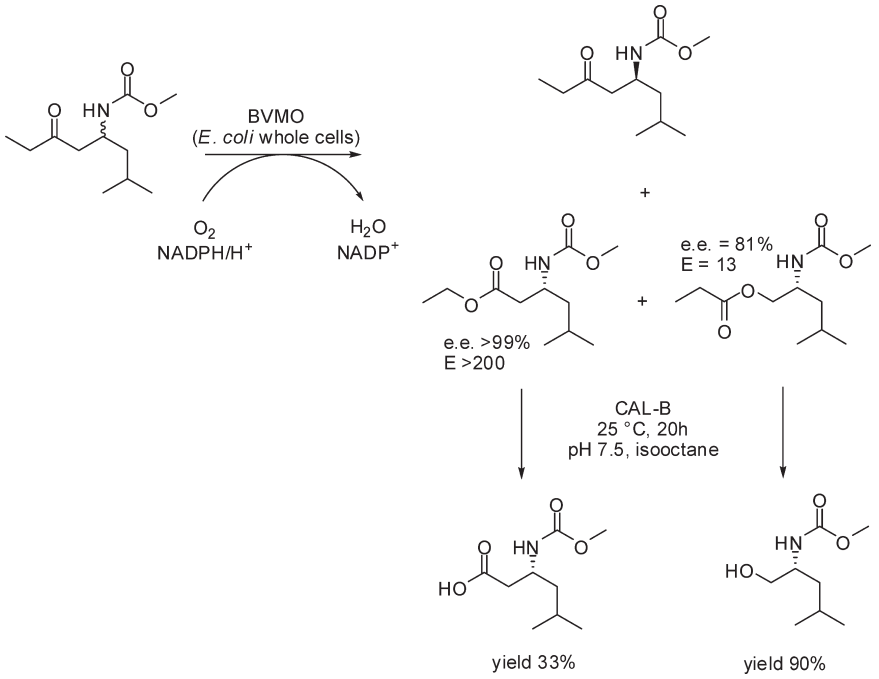

Figure 25. BVMO-catalyzed oxidation of *N*-protected 5-amino-3-one followed by CAL-B hydrolysis of the ester moiety. Adapted from ref 150. Copyright 2010 Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

o-Xylene dioxygenase from *Rhodococcus* sp. strain DK17 was shown to oxidize *p*-xylene to *cis*-*p*-xylene dihydrodiol, while biphenyl and naphthalene were oxidized to *cis*-2,3-biphenyl dihydrodiol and *cis*-1,2-naphthalene dihydrodiol, respectively. The enzyme displayed regioselectivity dependent on the size and position of the substituent groups on the aromatic ring. Toluene and ethylbenzene were indeed hydroxylated at the 2,3 and the 3,4 positions in the ratios of 8:2 and 9:1.[162]

2.2.4. Outlook. The combination of various biocatalysts for the development of new synthetic routes to enantiopure compounds nowadays becomes more and more common, as enzymes are being better understood and characterized, allowing the identification of ideal process conditions that can be sustained by different types of redox catalysts.

In one recent example, a BVMO and an ADH were used in combination for parallel interconnected kinetic asymmetric transformations (PIKAT).[163] A set of racemic and prochiral compounds was converted in a one-pot reaction concurrently yielding enantio-enriched ketones, sulfoxides, and *sec*-alcohols in a strict parallel fashion. This methodology only employed a catalytic amount of cofactor as connector and minimum quantity of reagents, and maximized the redox economy of the whole process by coupling two asymmetric reactions (either two kinetic resolutions or a kinetic resolution and a desymmetrization reaction), from which at least one should be (quasi)irreversible to allow high conversion. Through careful selection of the biocatalysts, all enantiomers could be obtained by interconnecting the two asymmetric transformations. For instance, parallel interconnected kinetic resolution of racemic 2-phenylpentan-3-one and racemic sulcatol was performed by combining PAMO and an ADH from *Lactobacillus brevis* (Figure 26). Three optically active products were obtained in high enantiomeric excess ((*R*)-2-phenylpentan-3-one, (*S*)-1-phenylethylpropionate, and (*S*)-sulcatol in 98%, 96%, and 99% *ee*, respectively), while conversion was limited to 50% (typical example of kinetic resolution) with traces (2%) of 4-methylpent-3-enylacetate as byproduct of the BVMO-catalyzed oxygenation of sulcatone.[163] The PIKAT method has also been applied to the stereoselective oxidation of prochiral sulfides and kinetic resolution of racemic 2-octanol, employing BVMOs and ADHs.[164]

2.3. Engineering Techniques

The implementation of biocatalysts in an increasing number of industrial synthetic routes (see section 4) relies not only on their exquisite natural regio-, stereo- and enantioselectivities but also mostly on the contribution of various engineering methods to render enzymatic processes more competitive. During the past decade, tremendous progress has been made in protein engineering techniques, allowing the improvement of a given enzyme for large-scale applications. Excellent reviews abound in this field[165−171] and successful examples can be found herein.

The engineering of strains has also proved highly valuable in the creation of tailor-made redox biocatalysts, as was shown for the synthesis of optically active alcohols using "designer cells" coexpressing the desired ADH and recycling enzyme for cofactor regeneration (see section 3). A single plasmid was used for overexpression of both proteins in *E. coli* and the system operated at high substrate concentrations (>100 $g \cdot L^{-1}$) with no addition of external cofactor required. Reduction of various ketones to the corresponding alcohols proceeded with high conversions (>90%) and enantioselectivity (>99.8%) in pure aqueous medium. 4-Chloroacetophenone for instance was reduced on a 10-L scale (substrate titer 156 $g \cdot L^{-1}$, wet biomass in 25 $g \cdot L^{-1}$) and subsequent extraction yielded 91% of 1-(*R*)-(*p*-chlorophenyl)-ethanol (>99.8% ee) in high purity (95%).[172]

Reaction engineering techniques are also being applied to overcome poor substrate solubility or product inhibition. In one example, efficient whole-cell biotransformation was achieved in a biphasic ionic liquid/water system using BMIM[Tf_2N]. *Lactobacillus kefir* cells were employed in a 200-mL batch process

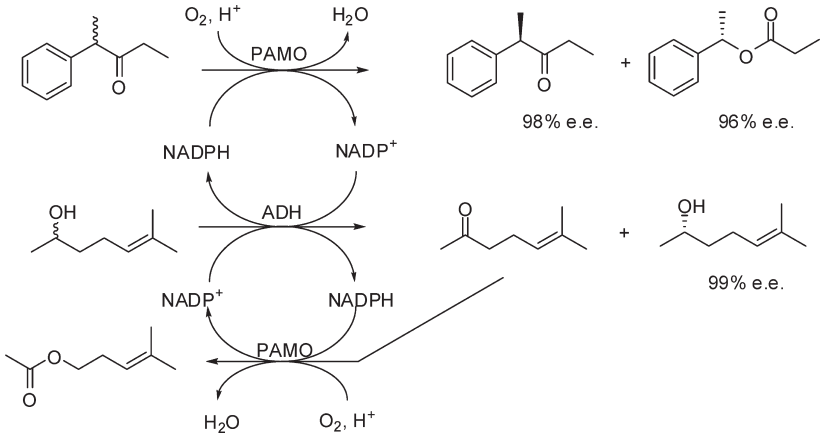

Figure 26. Concurrent kinetic resolution employing BVMO and ADH via PIKAT method. Adapted from ref 163. Reproduced by permission of the Royal Society of Chemistry.

for the reduction of 4-chloroacetophenone. High volumetric productivity (20.4 $g \cdot L^{-1}$) and chemical yield (93.8%) were obtained without addition of cofactor as the cellular cofactor regeneration system was still active in presence of the ionic liquid.[173] More recently, water miscible ionic liquids were used for the stereoselective reduction of hardly water-soluble aliphatic ketones by ADH from *Lactobacillus brevis*. Addition of AMMOENG 101 to the buffer system (10% v/v) led to higher activity on 2-octanone and higher stability of both ADH and GDH (used for cofactor recycling).[174]

Overall, all these techniques have contributed to the development of biocatalytic systems from originally wild-type strains to isolated enzymes and to engineered proteins and strains.

3. COFACTOR REGENERATION

The use of oxidoreductases for cofactor regeneration has reached a high standard, where cofactor cost is no longer an issue. Highly effective regeneration methods are now available and are currently applied to various redox processes. Traditional methods are the coupled-substrate approach and the coupled-enzyme approach. While a single enzyme and a cosubstrate are required in the former, an additional recycling enzyme together with its specific cosubstrate is used in conjunction with the enzyme catalyzing the reaction of interest in the latter.[15,16,26,28] New trends in nicotinamide cofactor regeneration have been recently reviewed.[175,176]

3.1. Alcohol Dehydrogenase

Alcohol dehydrogenase holds a special position among enzymes for a reductive production reaction because it can act as its own regeneration enzyme as well (see section 2.1.3.1). In the coupled-substrate approach,[13,177−183] isopropanol is employed to regenerate NAD(P)H from $NAD(P)^+$. The resulting acetone is stripped via pervaporation.

In the coupled-enzyme approach, ADH has been used for the regeneration of nicotinamide cofactor consumed in oxidation reactions. The PAMO-catalyzed oxygenation of phenylacetone to the corresponding lactone was chosen by Reetz et al. as model system and the cofactor recycling was accomplished by using a thermostable ADH from *Thermoanaerobacter ethanolicus* and isopropanol as sacrificial hydrogen donor. A two-liquid phase system was successfully applied to various ketones and high turnover numbers were obtained (TON > 30 000).[184] In another example, ADH from *Lactobacillus brevis* was coexpressed in *E. coli* with a cytochrome P450 monooxygenase to perform steroid hydroxylation reactions (Figure 27). Progesterone and testosterone were converted to the corresponding 15β-hydroxylated products with productivities up to 5.5 $g \cdot L^{-1} \cdot d^{-1}$ using crude cell extracts.[185]

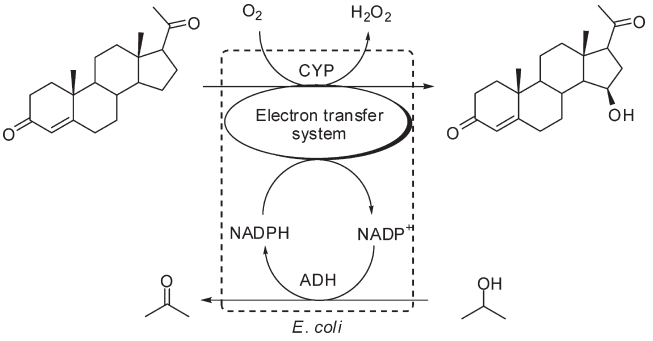

Figure 27. Specific hydroxylation of progesterone using *E. coli* crude cell extracts coexpressing CYP, ADH, and an electron transfer system (bovine adrenodoxin Adx and adrenodoxin reductase AdR). Adapted from ref 185. Copyright 2010 Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

3.2. Formate Dehydrogenase

Formate dehydrogenase (FDH, EC 1.2.1.2) catalyzes the practically irreversible oxidation of formate to carbon dioxide, with concomitant reduction of NAD^+ to NADH. As this reaction utilizes an inexpensive substrate, sodium or ammonium formate, and just leaves a gaseous by-product, CO_2, FDH has been widely employed for the regeneration of NAD^+ to NADH, such as in the syntheses of *tert*-leucine from trimethylpyruvate via leucine DH catalysis,[186] of allysine ethylene acetal, a building block of Omapatrilat, with PheDH,[187] or of (*R*)-3-(4-fluorophenyl)-2-hydroxy propionic acid with lactate DH, a building block for Rupintrivir, a rhinovirus protease inhibitor to treat the common cold.[188]

FDH has been characterized from *Pseudomonas sp. 101*,[189] from *Mycobacterium vaccae*,[190] from *Candida methanolica*,[191] and especially from *Candida boidinii*.[192] A variant of FDH from *C. boidinii* has been developed which can also accept $NADP^+$ as a substrate;[193] later, the sites Asp195, Tyr196 and Gln197 were

dx.doi.org/10.1021/cr200013n |*Chem. Rev.* 2011, 111, 4088–4110

mutated exhaustively for further improvements,[194] while Gln223Asp mutant showed reverse coenzyme preference from $NADP^+$ to NAD^+.[195]

C. boidinii FDH has been stabilized against oxidation of labile cysteines: of the three residues potentially labile to oxidation identified via homology modeling on the solved structure of FDH from *Pseudomonas putida*,[196] Cys23, Met134, and Cys262, two much more stable variants, Cys23Ser, and Cys23Ser/Cys262Ala, were developed via site-directed mutagenesis.[197] FDH from *C. boidinii* has also been thoroughly characterized as to stability against gas−liquid interfaces, chaotropic salts, and temperature: generally, the same order of stability as against overoxidation was found.[198] While shear does not seem to harm the enzyme, gas−liquid interfaces, such as from gas bubbles, reduce both activity and folded protein proportionally to the amount of interface presented. Likewise, when conveyed through a centrifugal pump, known to cause cavitation, that is, gas/liquid- or vacuum/liquid interfaces, at the edges of the pump blades, FDH degrades in a first-order behavior with respect to the amount of fluids pumped (and active FDH contained in it).

FDH has been employed with other dehydrogenases in continuous membrane reactors, where both enzymes were retained behind an ultrafiltration membrane. In general, high space-time yields, around 560 $g \cdot L^{-1} \cdot d^{-1}$[188] or 638 $g \cdot L^{-1} \cdot d^{-1}$ for trimethylpyruvate/LeuDH, and high total turnover numbers, 600 000 and 125 000 with phenylpyruvate/PheDH and trimethylpyruvate/LeuDH, respectively, have been obtained.[98,199]

Efficient biocatalytic process for the preparation of *sec*-alcohols was developed by using recombinant *E. coli* coexpressing an alcohol dehydrogenase from *Pichia finlandica* (PfODH) and FDH from *Mycobacterium* for the production of ethyl-(*S*)-4-chloro-3-hydroxybutanoate ((*S*)-ECHB), a key intermediate in the synthesis of inhibitors of HMG-CoA reductase and antibiotics. Ethyl 4-chloroacetoacetate (ECAA) was reduced to (*S*)-ECHB at 32.2 $g \cdot L^{-1}$ and 98.5% yield with 99% *ee* with cofactor regeneration.[200]

3.3. Phosphite Dehydrogenase

Another nearly irreversible reaction is the NAD^+-catalyzed oxidation of phosphite to phosphate via phosphite dehydrogenase (PTDH).[201]

PTDH has been reengineered for relaxed cofactor specificity. Through homology modeling, Glu175 and Ala176 in *Pseudomonas stutzeri* were identified as possible determinants of cofactor specificity. The single and double mutants E175A and A176R showed significantly better catalytic efficiency toward both cofactors; the double variant had a 3.6-fold and 1000-fold improved catalytic efficiency for NAD^+ and $NADP^+$, respectively. The double variant preferred $NADP^+$ 3-fold over NAD^+.[202]

With the help of PTDH-E175A/A176R and NADP-dependent xylose reductase, xylose was efficiently converted into xylitol at approximately four times the rate of wt-PTDH or even NADP-specific FDH from *Pseudomonas* sp.101.[203]

The enzyme from *Pseudomonas stutzeri* was thermostabilized during three rounds of random mutagenesis and high-throughput screening, after which a total of 12 thermostabilizing amino acid substitutions were identified. When combined, the variant featured a half-life of thermal inactivation at 45 °C of more than 7000-fold greater than and a T_{50} value 20 °C higher than the parent.[204] The stabilized and cofactor-relaxed phosphite dehydrogenase variant (12x-A176R PTDH) from *Pseudomonas stutzeri* was employed with xylose reductase to synthesize xylitol from xylose and with ADH, (*R*)-phenylethanol from acetophenone in an enzyme membrane reactor. The space-time yields of xylitol with the mutant PTDH were up to 4-fold increased over values with the $NADP^+$-specific *Pseudomonas* sp.101-FDH, up to 230 $g \cdot L^{-1} \cdot d^{-1}$ xylitol when using a charged nanofiltration membrane.[205]

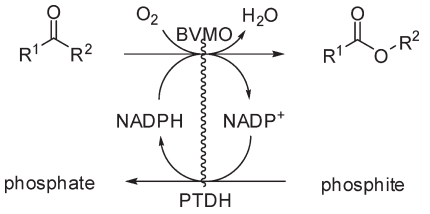

Figure 28. Synthesis of lactones from ketones with NADPH regeneration using a self-sufficient BVMO fused to a PTDH. Adapted from ref 206. Copyright 2008 Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

PTDH has been employed in a novel and elegant approach to cofactor regeneration, where it was fused to the enzyme performing the catalysis reaction. A number of representative BVMOs were covalently linked to soluble NADPH-regenerating phosphite dehydrogenase. This self-sufficient BVMO allowed the use of phosphite as a cheap and sacrificial electron donor with whole cells, cell extracts, and purified enzyme, and was shown to efficiently catalyze the stereoselective desymmetrization of various ketones to furnish enantiopure lactones (Figure 28).[206,207] The fusion protein approach to cofactor regeneration works best if both production and regeneration enzymes possess approximately the same specific activity.

3.4. Hydrogenase

The simplest regeneration scheme would allow for molecular hydrogen to be disproportionated to hydride, reducing NAD(P)$^+$ to NAD(P)H, and a proton. No other by-product besides the proton would be formed. Hydrogenase (H_2:$NADP^+$ oxidoreductase, EC 1.18.99.1) does achieve this task.

The soluble enzyme from the marine hyperthermophilic archaeon *Pyrococcus furiosus* exhibits sufficiently high stability under reaction conditions. In two repetitive batch-series 6.2 $g \cdot L^{-1}$ NADPH were produced with a total turnover number (mol produced NADPH/mol consumed enzyme) of 10 000. Combined with a NADPH-dependent thermophilic ADH from *Thermoanaerobium* sp. (ADH M) in an enzyme-membrane reactor, both acetophenone and (2*S*)-hydroxy-1-phenyl-propanone (HPP) were quantitatively reduced to (*S*)-phenylethanol and the (1*R*,2*S*)-diol with *ee* >99.5% and *de* >98% and ratios of product to cofactor $NADP^+$ of 100 and 160, respectively (Figure 29).[208]

3.5. Pyridine Nucleotide Transhydrogenase

In living whole-cell biocatalysts or in situations where both NAD(H) and NADP(H) are required, such as for different parts of a complex regeneration scheme, often one cofactor is more rapidly depleted than the other one. In that situation, a pyridine nucleotide transhydrogenase (PNT) can equilibrate the levels of NAD(H) and NADP(H).

The pntA and pntB genes from *E. coli* were cloned and coexpressed with $NADP^+$-dependent ADH from *Lactobacillus kefir* and NAD^+-dependent FDH from *Candida boidinii*. With this whole-cell biocatalyst, superior performance was demonstrated

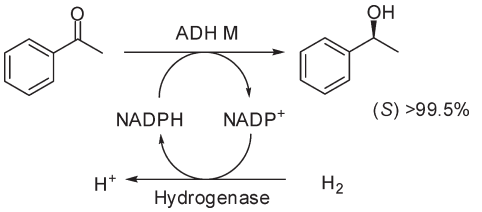

Figure 29. NADPH regeneration using a hydrogenase in the ADH-catalyzed reduction of acetophenone to (*S*)-phenylethanol. Adapted from ref 208, Copyright 2003, with permission from Elsevier.

compared to the case without PNTs: acetophenone was reduced to (*R*)-phenylethanol in 66% yield over 12 h, whereas only 19% (*R*)-phenylethanol was formed under the same conditions with cells containing ADH and FDH genes but without PNT genes. Both cofactors need to be present to yield alcohol product.[209]

Earlier, the soluble PNT from *Pseudomonas fluorescens* was employed in a cell-free system for the regeneration of both $NADP^+$ and NAD^+, resulting from the $NADP^+$-dependent morphine dehydrogenase and the NADH-dependent morphinone reductase, respectively, during the synthesis of the opiate drug hydromorphone (Figure 30). The ratio of morphine dehydrogenase, morphinone reductase, and PNT was critical for diminishing the production of the unwanted byproduct dihydromorphine and for optimum hydromorphone yields. Application of the soluble pyridine nucleotide transhydrogenase to the whole-cell system resulted in an improved biocatalyst with an extended lifetime.[210]

Lastly, NADPH was utilized to catalyze the oxidation of a model substrate (*p*-nitrophenoxydecanoic acid) via cytochrome P450 BM3 and was regenerated by a two-step cofactor regeneration constructed by NAD^+-dependent bacterial glycerol dehydrogenase (GLD) and the bacterial soluble transhydrogenase, both from *E. coli*. NADH was regenerated by GLD from the oxidized cofactor (NAD^+) using glycerol as a sacrificial cosubstrate. The reducing equivalents then were transferred to $NADP^+$ by PNT. In presence of the two-step cofactor regeneration, 50 μM *p*-nitrophenoxydecanoic acid was completely converted within 1 h using 5 μM of oxidized cofactors NAD^+ and $NADP^+$. In contrast, the system lacking the two-step cofactor regeneration gave just 34% conversion of 50 μM substrate using 50 μM NADPH.

3.6. Glucose Dehydrogenase

Glucose dehydrogenase (GDH) features several advantages that explain its favored use in regeneration: the enzyme accepts both NAD^+ and $NADP^+$, often equally well, and with a high substrate specificity of up to 550 $U \cdot mg^{-1}$. Glucose is a very inexpensive substrate and the product, gluconolactone, spontaneously hydrolyzes to gluconic acid, providing a favorable equilibrium.

GDH from *B. subtilis* is activated by high ionic strength.[211] However, variants hugely thermostabilized through either directed evolution[212−214] or via structure-guided consensus[215] not only were also stable against water-miscible organic solvents but also turned out to be less sensitive to changes in salt levels.[216] Interestingly, the decisive mutations for thermostabilization, E170K/R and Q252L, were achieved with both combinatorial as well as data-driven protein engineering.

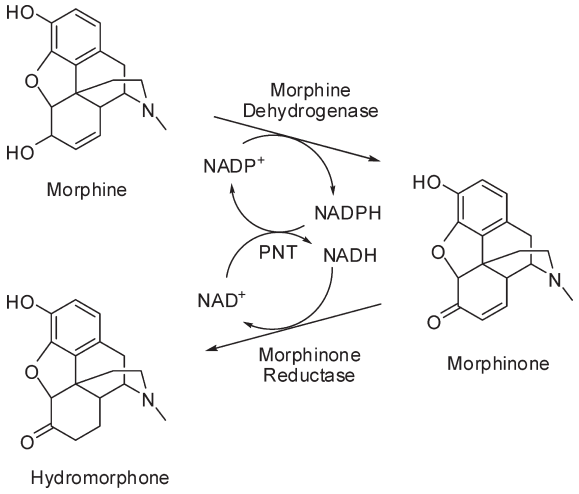

Figure 30. Synthesis of hydromorphone from morphine using pyridine nucleotide transhydrogenase (PNT) for cofactor regeneration. Adapted with permission from ref 210. Copyright 2000 ASM.

GDH has been employed for regeneration in various forms, as a soluble enzyme and in whole-cells. Also, GDH and the production enzyme, here glycerol DH, have been coexpressed in *E. coli* in a synthesis of L-glyceraldehyde.[217]

3.7. Amino Acid Dehydrogenase and Amino Acid Oxidase

The asymmetric transformation of a ketone to an amine formally counts as a stereoselective reductive amination. However, the production reaction was catalyzed by an ω-transaminase to afford a primary ω-chiral unprotected amine. The coproduct, pyruvate, was regenerated via catalysis with alanine dehydrogenase (AlaDH) and FDH, with formate as the ultimate reducing agent. This cascade only requires formate and ammonia as cosubstrates besides the ketone.[218]

An equivalent cascade has been set up in the opposite direction with an oxidative production reaction.[219] (*S*)- as well as (*R*)-mexiletine [1-(2,6-dimethylphenoxy)-2-propanamine], a chiral orally effective antiarrhythmic agent, was obtained through deracemization of the racemate in up to >99% *ee* and conversion with 97% isolated yield applying an ω-transaminase. The cosubstrate pyruvate needed in the first oxidative step was recycled from alanine by using an amino acid oxidase. The configuration of the product was determined by applying either the (*S*)- or the (*R*)-transaminase first.

3.8. NADH Oxidase

While the use of dehydrogenases in reductive reactions is well developed, their use for oxidative reactions is much less so. However, especially with increased use of renewables, chemo-, regio-, and enantioselective oxidation of alcohols, polyols, hydroxyl acids, or amino acids is an important capability. The use of dehydrogenases in organic synthesis benefits from their often superb enantioselectivity to prepare enantiomerically pure alcohols, hydroxy acids, or amino acids, from racemates but requires regeneration of NAD(P)(H) cofactor for economic syntheses. This regeneration advantageously can be afforded by NADH oxidase: the enzyme employs abundant, inexpensive, and environmentally benign molecular oxygen to regenerate $NAD(P)^+$ from NAD(P)H with concomitant reduction of oxygen to H_2O_2 or, more commonly, to innocuous H_2O. The net reaction of the

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oxidation of an alcohol moiety to a ketone or aldehyde is written as follows:

$$\text{dehydrogenase reaction}: \quad \text{R-CH}_2\text{OH} + \text{NAD}^+ \rightarrow \text{R-CHO} + \text{NADH} + \text{H}^+$$

$$\text{NADH reaction}: \quad \text{NADH} + \text{H}^+ + \frac{1}{2}\text{O}_2 \rightarrow \text{NAD}^+ + \text{H}_2\text{O}$$

$$\text{net reaction}: \quad \text{R-CH}_2\text{OH} + \frac{1}{2}\text{O}_2 \rightarrow \text{R-CHO} + \text{H}_2\text{O}$$

A platform of coupling dehydrogenases with NAD(P)H oxidases offers several advantages over similar systems: the irreversible NADH-catalyzed production of water shifts the unfavorable thermodynamics of the dehydrogenase-catalyzed oxidation and thus drives the overall reaction to completion; the broad specificity and superior chemo-, regio-, and enantioselectivity of dehydrogenases leaves optimization of conversion as the main problem, not selectivity; neither complex or expensive oxidants, nor metals or halogenated compounds are employed.

At least six bacterial water-forming NAD(P)H oxidases (nox2) have been developed, from *Streptococcus faecalis*,[220] *Streptococcus mutans*,[221] *Streptococcus pyogenes*,[222] *Lactobacillus sanfranciscensis*,[223] *Lactobacillus brevis*,[224] and from *Lactococcus lactis*.[225] Recently, another, very stable homologue was characterized from *Lactobacillus plantarum*.[243] The enzyme from *Lactobacillus sanfranciscensis* is unique in accepting either NADH or NADPH, thus broadening the range of dehydrogenases.[226]

Located next to the flavin cofactor, water-producing NADH oxidases feature a single cysteine residue which cycles between free thiolate and sulfenic acid during catalysis.[227,228] NADH oxidases have been found to be turnover-limited, i.e. they produce a roughly constant amount of product per active site during the lifetime. In presence of reducing agents such as DTT (dithiothreitol), the *L. sanfranciscensis*-nox2 has a very high total turnover number (TTN) of >125 000, that is, more than 10^5 moles of product are generated per mole of enzyme active site or of cofactor, but only a TTN of 5000 in the absence of DTT. The difference for the *L. lactis* enzyme is far smaller: 78 000 vs 38 000.[225]

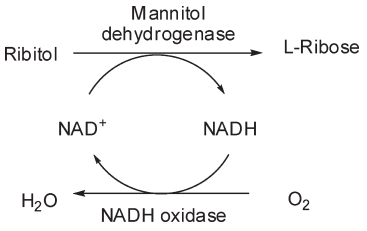

Figure 31. Schematic conversion of ribitol to L-ribose through mannitol-1-dehydrogenase from *Apium graveolens*[230] complemented with NADH cofactor regeneration using NADH oxidase.

Examples include the synthesis of D-*tert*-leucine from D,L-*tert*-leucine, of α-keto glutarate from L-glutamate, and last the synthesis of specialty L-sugars from carbohydrates.

Just as (*S*)-*tert*-leucine, a building block for HIV protease inhibitors and matrix metalloprotease inhibitors (MMPIs), is difficult to obtain from precursors,[92] a workable synthesis to (*R*)-*tert*-leucine was elusive until the (*S*)-enantiomer of the racemate could be oxidized to trimethylpyruvate with catalysis by LeuDH/NADH oxidase from *L. brevis*.[224]

α-Keto glutarate is effective against mild kidney insufficiency and can be synthesized by simple oxidation of L-glutamate, available inexpensively as monosodium glutamate (MSG), a taste enhancer used preferentially in Eastern Asian cuisines. Oxidation by MSG to α-keto glutarate catalyzed by glutamate dehydrogenase (GluDH)/NADH oxidase (from *L. sanfranciscensis*) proceeds to completion but with very low space-time yield, owing to substrate and product inhibition of GluDH.[229]

Rare sugars, such as L-mannose, L-ribose, and L-xylose, are known to be possible building blocks for anticancer drugs such as Emtricitabine and Clevudine. Since they cannot be produced by fermentation, an alternative method to synthesize these molecules is needed. Several forms of dehydrogenases, which utilize nicotinamide-based cofactor NAD(P)$^+$, have been found to have the ability to synthesize these rare sugars. Consequentially, the need of enzyme that would regenerate NAD(P)$^+$ from NAD(P)H has arisen. By utilizing enzymes that catalyze selective oxidations of carbohydrates and regenerate the cofactor NAD(P)$^+$, the metabolic pathway could be brought to completion and the economic efficiency increased (Figure 31).

3.9. Others

A recent case with ene-reductases was reported where the asymmetric reduction of various activated olefins did not require the recycling of the nicotinamide cofactor (cofactor free system).

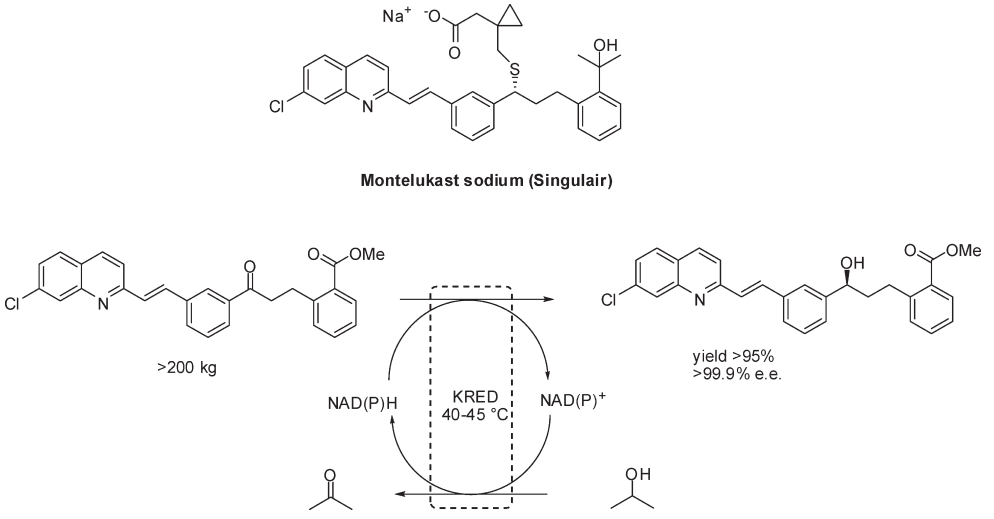

Figure 32. Codexis biocatalytic step in the synthesis of montelukast. Adapted with permission from ref 179. Copyright 2010 American Chemical Society.

In this study, advantage was taken of the ability of OYE homologues to catalyze the disproportionation of enones: while one molecule was reduced by the flavin, another molecule was oxidized by the oxidized flavin and subsequently underwent spontaneous tautomerization to the corresponding phenol, thus driving the reaction toward reduction of the first enone. So far, the best example was obtained with the reduction of *N*-phenyl-2-methylmaleimide using 1,4-cyclohexanedione as H donor and YqjM as biocatalyst (51% conversion and >99% *ee* for (*R*)-*N*-phenyl-2-methylsuccinimide). This is a striking example of a nicotinamide-independent hydride transfer using enzymes that are usually nicotinamide-dependent and relying on a sacrificial H donor such as 2-enones or 1,4-diones.[231]

4. CASE STUDIES

As was shown in the previous sections, the use of biocatalysts affords the synthesis of chiral intermediates with high enantiopurity. Their advantages, when combined with classical chemical protocols, allows the development of powerful chemo-enzymatic routes that are currently being implemented for the generation of various pharmaceuticals. Examples in this field have been reviewed,[98,232−235] and the most prominent cases using enzymatic redox processes in large-scale API syntheses are detailed in this section.

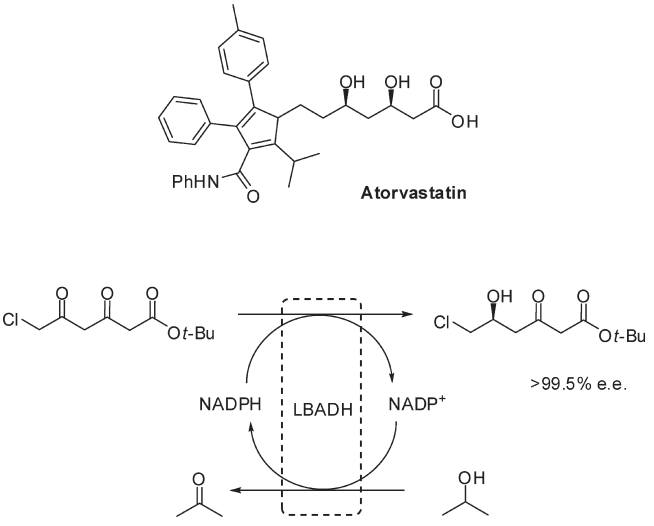

Figure 33. Enzymatic reduction using ADH from *Lactobacillus brevis* for the synthesis of a Lipitor building block. Adapted from ref 178. With kind permission from Springer Science + Business Media: Wolberg, M.; Villela, M.; Bode, S.; Geilenkirchen, P.; Feldmann, R.; Liese, A.; Hummel, W.; Muller, M. Chemoenzymatic synthesis of the chiral side-chain of statins: Application of an alcohol dehydrogenase catalysed ketone reduction on a large scale. *Bioprocess Biosyst. Eng.* **2008**, *31*, 183, Scheme 6.

4.1. Montelukast

Codexis has developed a new biocatalytic process for the synthesis of the key intermediate used in the production of montelukast ((*R*)-isomer), the active ingredient in Singulair, Merck's antiasthma drug. A ketoreductase (KRED) was engineered via directed evolution to catalyze the asymmetric reduction of (*E*)-methyl 2-(3-(3-(2-(7-chloroquinolin-2-yl)vinyl)phenyl)-3-oxopropyl)benzoate to the corresponding (*S*)-alcohol used in the synthesis of montelukast (Figure 32). The new KRED could work in the presence of 70% organic solvent at 45 °C to accept the water-insoluble substrate (100 $g \cdot L^{-1}$), while being highly regio- and stereoselective. The process is now currently run at >200 kg scale where the product is obtained in >95% yield in >99.9% *ee* and >98.5% chemical purity and offers a greener alternative to the process initially developed by Merck and based on a (−)-B-chlorodiisopinocampheylborane (i.e., (−)-DIP-Cl or (−)-Ipc2BCl)-mediated asymmetric reduction. In addition to featuring lower cost and generating far less waste, the enzyme-catalyzed step produced the desired alcohol in greater yield and higher stereochemical purity, employing KRED-catalyzed oxidation of isopropanol to acetone for regeneration of the cofactor. By screening several NAD(P)-dependent ketoreductases from available panel, some were found active on the substrate, displayed great stereoselectivity ((*S*)-product with *ee* >99.9%) but had low activity, were unstable at high organic solvent concentration and needed to be improved. Directed evolution techniques allowed a 3000-fold improvement in activity to

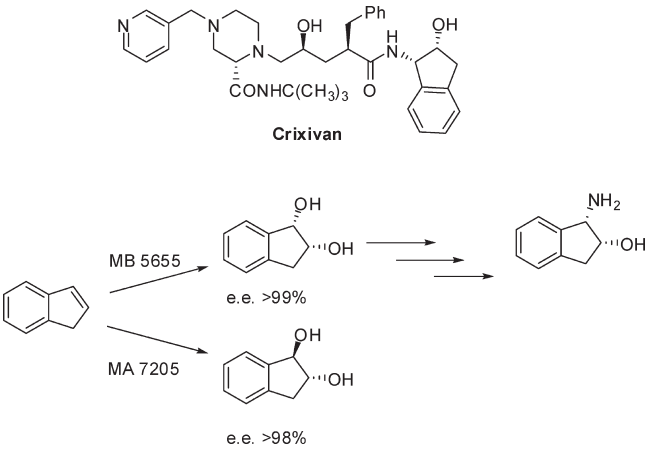

Figure 35. Bioconversion of indene to a chiral intermediate in Crixivan synthesis using *Rhodococcus* sp. strains. Adapted from ref 241, Copyright 1998, with permission from Elsevier.

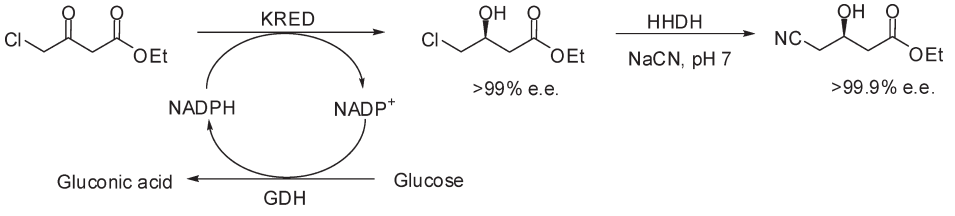

Figure 34. Codexis process for the production of a key intermediate in atorvastatin synthesis. Adapted with permission from ref 240, Copyright 2009 Elsevier, and from ref 236, Copyright 2007 Nature Publishing Group.

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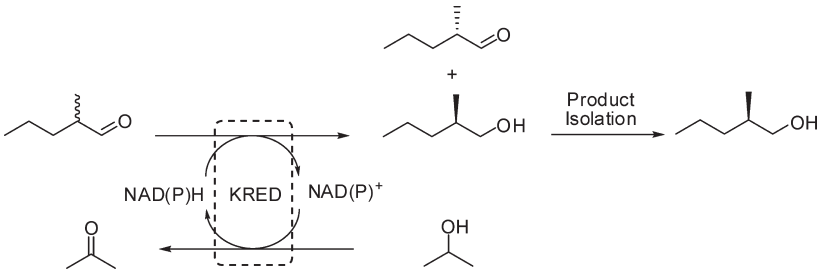

Figure 36. Enantiospecific reduction of 2-methylvaleraldehyde to (*R*)-2-methylpentanol. Adapted with permission from ref 183. Copyright 2010 American Chemical Society.

produce the final biocatalyst with 19 mutations obtained from the parent enzyme (and guided with ProSAR[236]).[179]

4.2. Lipitor

Lipitor (atorvastatin calcium) was the first drug to reach the annual sales of 10 billion dollars in the U.S.A. and is currently the top selling pharmaceutical product in the world.[237] The statin drug is a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor used to decrease levels of low density lipoprotein (LDL) cholesterol. The synthesis of the (3*R*,5*S*)-dihydroxyhexanoate side chain requires highly specific synthesis as high *ee* and *de* values are required (99.5% and 99%, respectively[238]), encouraging the development of various biocatalytic routes to achieve these challenging goals.[237]

In one example, a hydroxy ketoester intermediate to atorvastatin was obtained through the highly regio- and stereoselective single-site NADPH-dependent reduction of a β,δ-diketo ester using an ADH from *Lactobacillus brevis* (LADH). *tert*-Butyl 6-chloro-3,5-dioxohexanoate was converted in 72% yield to enantiopure *tert*-butyl (*S*)-6-chloro-5-hydroxy-3-oxohexanoate (Figure 33). 2-Propanol was used for cofactor regeneration as LADH catalyzes the oxidation of the alcohol to acetone at the expense of $NADP^+$, while simultaneously improving the solubility of the lipophilic substrate in the aqueous phase. A detailed kinetic study allowed improvement of the cofactor consumption by decreasing the amount of $NADP^+$ 60-fold while the cofactor total turnover number was improved 50-fold. The reaction was successfully scaled-up to 100 g.[178,180,239]

Another route developed by Codexis involves both a ketoreductase (KRED) and a halohydrin dehalogenase (HHDH), starting from 4-chloroacetoacetate and giving access to (*R*)-4-cyano-3-hydroxybutyrate as key intermediate (Figure 34). Codexis won the 2006 Presidential Green Chemistry Challenge Award from the United States Environmental Protection Agency (USEPA) under the focus category "Greener Reaction Conditions" for the development of this process.[236,240]

Some other routes have already been mentioned earlier in this review (see section 2.1.3.3).

4.3. Crixivan

Biocatalytic routes to Crixivan, an HIV-protease inhibitor, have been developed based on oxygenases. A key chiral synthon for Crixivan is *cis*-(1*S*,2*R*)-1-aminoindan-2-ol and potential precursors include *cis*-(1*S*,2*R*)-indandiol and *trans*-(1*R*,2*R*)-indandiol (Figure 35). Two *Rhodococcus* sp. strains were found to contain a toluene dioxygenase yielding the cis-isomer diol and both a toluene dioxygenase and a naphthalene monooxygenase producing the trans-isomer diol. Strain MB5655 produced up to 2.0 $g \cdot L^{-1}$ of the cis-isomer (*ee* >99%), while strain MA 7205 had a slightly lower productivity and stereoselectivity (1.4 $g \cdot L^{-1}$ of *trans*-(1*R*,2*R*)-indandiol at *ee* >98%).[241] Bioprocess engineering allowed the production of *cis*-(1*S*,2*R*)-indandiol in up to 200 $mg \cdot L^{-1} \cdot h^{-1}$.[242]

4.4. (*R*)-2-Methylpentanol as Building Block

A process to prepare enantiopure (*R*)-2-methylpentanol, an important intermediate for the production of certain pharmaceuticals and natural products, has been developed by Codexis and Pfizer. The kinetic resolution of racemic 2-methylvaleraldehyde was performed using a ketoreductase from *Lactobacillus kefir* in a substrate-coupled approach for recycling of the nicotinamide cofactor. KRED was evolved to improve both its selectivity toward the (*R*)-enantiomer and its volumetric productivity (Figure 36). After three rounds of evolution, a biocatalyst (total of five mutations) meeting all of the targeted process conditions was obtained and was used in a pilot run in 400 kg substrate charge. 45% conversion was achieved within 28 h affording nearly 100 kg of product in high enantiopurity (98.2% *ee*). EDTA was added after 45% to stop the reaction and prevent the formation of the (*S*)-enantiomer.[183]

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BIOGRAPHIES

Mélanie Hall (born 1980 in Brest, France) obtained her Master's degree in chemistry at the Ecole Nationale Supérieure

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Andreas Bommarius is a Professor in the Schools of Chemical & Biomolecular Engineering and Chemistry/Biochemistry at the Georgia Institute of Technology in Atlanta, GA, U.S.A., since 2000. He obtained degrees in Chemistry (diploma at the Technical University of Munich, Germany in 1984) and in Chemical Engineering (Massachusetts Institute of Technology, Cambridge, MA, U.S.A., B.S. 1982 and PhD 1989). Until 2000, he headed the Enzyme Catalysis lab and pilot plant of Degussa (now Evonik) in Wolfgang, Germany. His research interests focus on biocatalysis and bioprocessing, more specifically on the development of novel biocatalysts, protein stability, and data-driven protein engineering. Since February 2010, he is the Director of the NSF I/UCRC Center for Pharmaceutical Development (CPD).

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